Cardiovascular and metabolic effects of dietary selenomethionine exposure in fishes

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Toxicology Graduate Program,

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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ABSTRACT

Selenium (Se) is an essential micronutrient involved in important metabolic functions for all vertebrate species. As Se is reported to have a narrow margin between essentiality and toxicity, there is growing concern surrounding the adverse effects of elevated Se exposure caused by anthropogenic activities. Recent studies have reported that elevated dietary exposure of fish to selenomethionine (Se-Met) can alter aerobic metabolic capacity, energetics and swimming performance. My thesis aimed to further investigate mechanisms of sublethal Se-Met toxicity, particularly potential underlying cardiovascular implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met in adult zebrafish (*Danio rerio*) and juvenile (yearling) rainbow trout (*Oncorhynchus mykiss*). In my first experiment, adult zebrafish were fed either control food (1.1 μg Se/g dry mass [d.m.]) or Se-Met spiked food (10.3 or 28.8 μg Se/g d.m.) for 90 d at 5% body weight per day. In the second experiment, juvenile rainbow trout were fed either control food (1.3 μg Se/g d.m.) or Se-Met spiked food (6.4, 15.8 or 47.8 μg Se/g d.m.) for 60 d at 5% body weight per day. Following exposure, ultrahigh resolution B-mode and Doppler ultrasound was used to characterize cardiac function. Chronic dietary exposure to elevated Se-Met had opposing results in zebrafish when compared to the rainbow trout. Zebrafish exposed to the highest dietary concentrations of Se-Met (28.8 μg Se/g d.m.) had significantly reduced ventricular contractile rate, stroke volume, and cardiac output, while Se-Met exposed rainbow trout had significantly greater stroke volume, ejection fraction, and cardiac output.

Following ultasonography, energy stores were measured via whole body (zebrafish) and liver, heart and muscle (rainbow trout) glycogen and triglyceride concentrations.

Zebrafish in the highest exposure group were observed to have greater whole body glycogen concentrations when compared to the control group, while rainbow trout exposed to Se-Met concentrations greater than 15.8 μg Se/g showed significant increases in both glycogen and triglycerides in liver relative to the control group. In addition, rainbow trout in the highest exposure group had significantly reduced capability of managing blood glucose levels as was evident after 48hrs in a glucose tolerance test.

Exposure to Se-Met significantly decreased mRNA expression of a key cardiac remodelling enzyme, matrix metalloproteinase 2 (MMP2), in adult zebrafish heart, however significantly increased it (MMP9) in rainbow trout heart. Selenomethionine significantly increased echodensity at the junction between atrium and ventricle in zebrafish, and these results combined with increased MMP2 expression are consistent with cardiac remodelling and fibrosis. However, rainbow trout did not show any fibrosis and also had a significant decrease in SERPINH mRNA abundance, a molecular chaperone essential for the post-translational folding of fibril-forming collagens. This, taken together with the increase in MMP9, suggests an anti-fibrotic response in the rainbow trout heart, compared to the fibrosis seen in the zebrafish, which could help explain why there were opposing cardiovascular results. Due to the anti-fibrotic response in rainbow trout, the heart was apparently able to pump blood more effectively leading to the increase in stroke volume, ejection fraction, and cardiac output observed.

The results of this study suggest that chronic exposure to dietary Se-Met can impact cardiac function, energy homeostasis and cause cellular perturbations, and such physiological consequences could reduce the aerobic capacity and survivability of fish.

The varying results seen could be attributed to species sensitivity differences or perhaps due to the cold vs warm water fish selenium sensitivity.

ACKNOWLEDGEMENTS

I would like to thank my graduate supervisors, Dr. David Janz and Dr. Lynn Weber for their guidance and mentorship during the completion of my degree. They have been instrumental in helping me achieve my goals and pursue my unending curiosity of the world of science. I would also like to thank my committee members, Dr. Patrick Krone and Dr. Barry Blakley for their support and advice. I must also thank my external examiner Dr. Keith Tierney for making his way here to attend my defense.

Thank you to Derek Green, Luciene Kapronczai and Anita Massé for their continuous support and positive motivation for me throughout both my experiments. I would also like to thank Zac Currie, Larry (larbear) D'Silva and Mike Cavallaro for being the best roommates one could ask for. The entire Toxicology Centre, from the front office staff (Tina, Fiona and Adriana) to the students and staff have been the absolute best and I could not have asked for a better group of people. I have made some of the best friendships and hope to hold on to these for many years to come. From conferences to beers nights, it was always a great time with this crew. I want to also thank my family for their unconditional love and support and for answering those mid-plating/mid-labelling/mid-sampling phone calls to hear about my day and what I ate for lunch. And most importantly, my partner in life Joanna Speed for always being by my side through the craziness of this Masters. Especially when I had to get up at all hours of the night to take blood samples from my fish, and helping me with the odd weekend water change.

I would like to finally acknowledge my funding sources for their financial support of this thesis. Funding was provided by the Government of Canada NSERC program and the Toxicology Centre.

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LIST OF ABBREVIATIONS

µg/g	Micrograms per gram
µg	Microgram
µg/L	Micrograms per litre
µm	Micrometre
ºC	Degrees centigrade
ANOVA	Analysis of variance
ATRF	Aquatic Toxicology Research Centre
BPM	Beats per minute
CAT	Catalase
cDNA	Complementary DNA
cm	Centimetre
CO	Cardiac output
CS	Citrate synthase
d	Day
dm	Dry mass
EF	Ejection fraction
EF1α	Elongation factor 1α
hERG	human ether-à-go-go-related gene
HOAD	β-hydroxyacyl coenzyme A dehydrogenase
g	Gram
GTT	Glucose tolerance test
GPX	Glutathione peroxidase
GST	Glutathione S transferase
ICP-MS	Inductively coupled plasma-mass spectrometry
kg	Kilogram
L	Litre
LOD	Limit of detection
m	Metre
MAT1A	Methionine Adenosyltransferase 1 α
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
mL	Millilitre
MMP2/9	Matrix metalloproteinase 2/9
MNE	Mean normalized expression
MO_2	Oxygen consumption
mm	Millimetre
mRNA	Messenger RNA
MS-222	Tricaine methanesulfonate
n	Number of samples
NSERC	Natural Sciences and Engineering Research Council of Canada
O_2	Oxygen
PAH	Polycyclic aromatic hydrocarbon
rt-PCR	Real-time polymerase chain reaction

Se	Selenium
Se0	Solid elemental selenium
Se-2	Inorganic selenide
Se+4	Selenite
Se+6	Selenate
SeCys	Selenocysteine
SelP	Selenoprotein P
SEM	Standard error of the mean
Se-Met	Selenomethionine
SERCA	Sarcoplasmic reticulum calcium ATPase
SERPINH	Serpin peptidase inhibitor, clad H1
SOD	Superoxide dismutase
SV	Stroke volume
TORT	Lobster hepatopancreas
USEPA	United States Environmental Protection Agency
XAS	X-ray absorption spectroscopy
XANES	X-ray absorption near-edge spectroscopy

NOTE TO READERS

This thesis was prepared in a manuscript style, and will therefore have some redundancies across sections of research chapters. To reduce these redundancies, specific descriptions of methods and statistics are found in their respective chapters. Chapter 1 is a general introduction, and chapters 2-3 are written in the style of publishable manuscripts. Chapter 4 serves as a summary and conclusion to the overall thesis. Chapter 2 of this thesis was published in Toxicological Sciences in October 2017, and chapter 3 has been submitted to Aquatic Toxicology and is in review. To avoid redundancies in citation lists, all citations have been provided in a combined section at the end of this thesis.

CHAPTER 1

1.0 GENERAL INTRODUCTION

Selenium (Se) is an essential micronutrient, involved in important metabolic functions for all vertebrate species. As it is reported to have a narrow margin between deficiency and toxicity (Janz et al., 2010), there is growing concern surrounding the adverse effects of elevated Se exposure. Early life stages of oviparous vertebrates, especially fishes, are highly susceptible to increased dietary Se supplementation (Hopkins et al., 2004; Thomas and Janz, 2011; Ohlendorf et al., 2011; Janz 2012). Selenium naturally enters aquatic environments in relatively low concentrations, providing fish and other aquatic organisms with adequate levels needed to maintain normal metabolic functions (Maher et al., 2010). However, anthropogenic activities such as coal, phosphate and uranium mining, as well as fertilizer use and coal-fired power plants cause elevated Se levels in aquatic environments (Maher et al., 2010; Janz et al., 2010). Using *Danio rerio* as a model test organism, a large body of evidence has shown that elevated dietary exposure to selenomethionine (Se-Met), the primary form of Se in the diet, can alter metabolic capacity, energy homeostasis, swimming performance and cause a greater incidence of juvenile deformities and mortalities (Tashjian et al., 2007; Thomas and Janz, 2011; McPhee and Janz, 2014). Conversely, there is a lack of laboratory research investigating the potential cardiovascular effects of elevated Se exposure. The overall objective of my thesis research is to investigate mechanisms of cardiovascular and metabolic dysfunction in fishes.

1.1 Selenium

Selenium (Se) is a metalloid element with the atomic number of 34 and a mass of 78.96. In the periodic table Se is in the same chalcogen group as oxygen, suphur, tellurium, and polonium, and is the 66th richest element in the earth's crust, with the greatest concentrations in cretaceous sedimentary rocks (Greenwood and Earnshaw, 1997; Presser et al., 2004).

1.1.1 Sources of selenium

Selenium is found across the globe and has varying concentrations throughout different regions depending on their geological structures (Maher et al., 2010). Selenium is commonly found in areas of sedimentary rock depositions, in particular phosphate, black shale and coal deposits (Presser et al., 2004; Maher et al., 2010). It enters the environment through natural weathering, biogeochemical processes and atmospheric deposition (Nriagu, 1989), however these contributing concentrations are considered minor compared to the role of anthropogenic activities (Maher et al., 2010). Petroleum refineries, mining and agricultural industries, as well as coal-fired power plants are all areas of economic importance that can greatly exacerbate the loading of Se into the environment by manufacturing waste by-product generation (Lemly, 2004; Maher et al., 2010; Janz, 2012). The adverse effects that high Se exposures can have on species health are well documented in laboratory experiments and are evident in numerous case studies, such as the Kesterson disaster. In the mid-80s, thousands of birds and fish were found severely deformed or dead in the Kesterson National Wildlife Refuge, following large-scale bioaccumulation of Se from agricultural runoff (Presser, 1994). This had huge

environmental ramifications, including the abandonment of 100,000 acres of once irrigated farmland in the San Joaquin Valley (Moore et al., 1990).

1.1.2 Selenium in aquatic environments

Selenium is a complex micronutrient that exists in one of four oxidation states: Se(-II), Se(0), Se(IV) and Se(VI) (Elrashidi et al., 1989). These compounds are found in both organic and inorganic forms. Under oxidizing conditions, Se forms multifaceted inorganic complexes, selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), which can readily be bioaccumulated and biotransformed by primary producers and microorganisms to form organic seleno-amino acid compounds, such as selenomethionine (Se-Met) and selenocysteine (Se-Cys) (Gladyshev, 2004; Orr et al., 2006). The ease for Se to bioaccumulate at the base of the food web is an area of concern as dietary organoselenium progresses through consumers at higher trophic levels, who integrate these seleno-amino acids into proteins, resulting in high concentrations of Se in upper predatory species, including fish (Furr et al., 1979; Hamilton, 2004). Selenium toxicity can have varying ecological effects depending on the hydrological, geological, and food web structure (Orr et al., 2006), and although controlled lab studies can contribute to the overall understanding, they are often unable to translate laboratory results to real ecosystem results (Landis and Yu, 1995). Past case studies such as the Kesterson Reservoir disaster, Belews Lake tragedy, the Hyco Reservoir and the Sweitzer Lake contamination (Baumann, 1986; Presser, 1994; Lemly, 2002) have helped shed light on the real environmental ramifications Se poisoning can have on aquatic organisms.

1.1.3 Selenium biochemistry

Selenium is known to be essential for proper metabolic functioning in all vertebrate species. Its essentiality was first recognized by the work of Schwarz and Foltz (1957), and has since gained a lot of attention due to its presence in important selenoproteins such as glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases (Lobanov et al., 2009; Gladyshev, 2012). Organisms in all three domains of life (eukaryotes, archaea, and eubacteria) utilize trace amounts of Se for the synthesis of selenoproteins (Xu et al., 2012). There are 23-25 known selenoproteins in mammals, while fishes possess one of the largest selenoproteomes with 30-37 known selenoproteins (Lobanov et al., 2009). Although the majority of these selenoproteins have unknown functions, the oxidoreductase enzymes mentioned above are some of the few that have been described (Janz et al., 2010). Using a reduced glutathione substrate, glutathione peroxidases (GPx) are highly efficient antioxidant enzymes that have a pivotal role in the reduction of lipid and hydrogen peroxide compounds (Baker et al., 1993; Bartoskova et al., 2014). In comparison, thioredoxin reductases are not only involved in resisting oxidative stress and maintaining the overall reducing environment of the cell, but also have a role in protein repair and DNA synthesis (Gladyshev, 2012). By removing specific iodine moieties, iodothyronine deiodinases convert thyroxine (T4) to active triiodothyronine (T3) and have vital metabolic roles, including thermogenesis, hearing and growth (Papp et al., 2007). Selenoprotein P is another well-documented selenoprotein and is estimated to bind 50% of the plasma Se, acting as the primary Se transport protein (Papp et al., 2007; Gladyshev, 2012).

The majority of Se exposure occurs through the diet via its organic form, selenomethionine (Se-Met) which easily bioaccumulates in aquatic food webs (Fan et al., 2002). Se-Met is biotransformed into selenide (Se^{2-}), which is then phosphorylated by selenophosphate synthetase and incorporated into desired selenoproteins via the selenocysteinyl-tRNA insertion sequence and the UGA codon (Suzuki and Ogra, 2002; Gladyshev et al., 2012). Due to the structural similarity between Se-Met and the amino acid methionine, Se-Met can avoid biotransformation and be directly integrated into any methionine-containing protein (Suzuki and Ogra, 2002). Because of this, we see in a dose-dependent manner increasing concentrations of Se-Met in tissues with high rates of protein synthesis including the liver, kidney, skeletal muscle, gonads as well as erythrocytes (Schrauzer, 2000). Given the antioxidant properties of Se, one would think increased concentrations would be beneficial, however excessive levels of Se-Met may actually compromise cellular redox homeostasis (Suzuki and Ogra, 2002).

1.1.4 Mechanisms of selenium toxicity

Several mechanisms have been proposed by which Se exerts its toxic effects, however only two hypotheses have ultimately gained support. The first proposed mechanism is based on the shared chemical similarities between sulphur and Se, thus giving Se the ability to substitute for sulphur during protein synthesis in a random manner (Maier and Knight, 1994). It is argued that this disrupts the disulfide S-S linkages and consequently alters the tertiary structure of the protein, potentially impairing its enzymatic activity (Sunde, 1984; Martinez et al., 2011). This hypothesis has however been contested as both methionine and selenomethionine have a terminal methyl group in their structure, thus preventing covalent bridge formation, and therefore having very

minimal influence on overall tertiary structure of a protein (Janz et al., 2010). For this reason alone it is unlikely that improper protein folding due to Se substitution is the mechanism behind Se toxicity.

The second, and more accepted hypothesis for Se-induced toxicity is due to oxidative stress (Palace et al., 2004; Janz et al., 2010) associated with glutathione homeostasis and its role as an intracellular antioxidant. Under normal conditions, glutathione peroxidases help maintain the overall reducing environment of the cell and prevent the formation of reactive oxygen species (ROS). The theory is that with increasing Se concentrations, there is a depletion of reduced glutathione, which leads to the production of ROS. This induces toxic effects including DNA and protein oxidation, lipid peroxidation and cellular apoptosis (Spallholz, 1994; Misra and Niyogi, 2009). Selenium metabolism leads to the formation of redox reactive intermediates, which react with glutathione and generate toxic hydrogen peroxide, superoxide anions and hydroxyl radicals (Spallholz et al., 1994; Spallholz et al., 2001). Moreover, the biotransformation of Se-Met to the highly nucleophilic and cytotoxic methylselenol further contributes to oxidative stress (Fernandes et al., 2012). This theory seems to be more plausible than its predecessor however, glutathione peroxidase may also play a role in the mechanisms behind Se toxicity. With increasing Se-Met concentrations, we see a dose-dependent increase in the concentrations of GPx, until it reaches a plateau. Following this, excess Se-Met is incorporated in a non-specific manner into proteins. Overexpression of GPx in mouse models leads to a massive increase in beta-cell mass, body fat content and the development of insulin resistance (McClung et al., 2004), potentially further contributing to adverse cardiovascular effects of Se toxicity.

1.1.5 Toxicological effects of selenium on oviparous species

A large body of research has recently been focusing on the toxicity of Se and its impact on aquatic biota. It is now well known that Se is extremely toxic to oviparous fish species, and can negatively impact the sustainability of wild fish populations (Maier and Knight, 1994; Skorupa, 1998). Following mercury, Se is considered as the second most toxic trace element to oviparous vertebrates (Luoma and Presser, 2009). Recent studies have reported that elevated dietary exposure of fish to Se-Met, the primary form of Se in the diet, can alter metabolic capacity, energy homeostasis, swimming performance and cause a greater incidence of early life stage deformities and mortality (Holm et al., 2005; Tashjian et al., 2007; Thomas and Janz, 2011; Thomas et al., 2013; McPhee and Janz, 2014). The most important toxicological effects of Se in fish arise from maternal transfer to embryos (Janz et al., 2010). Following exposure to elevated levels, maternal transfer of Se-enriched vitellogenin to developing oocytes leads to larval Se exposure during yolk resorption (Lemly, 1997; Janz, 2012). This exposure leads to numerous developmental deformities in juveniles such as spinal curvatures, craniofacial deformities, missing or deformed fins, and various forms of edema (pericardium or yolk sac edema) (Janz et al., 2010). Although not all deformities equally affect fish population viability, the consensus is that vertebral deformities are considered the most devastating as they affect the fishes' ability to swim to obtain food, find a mate or avoid predation (Koumondourous et al., 2002). Se-induced deformities and mortalities are also common amongst birds, however unlike fish, the maternal transfer of Se-containing proteins is in the albumin not yolk sac (Janz et al., 2010). This again causes significant embryonic deformities such as malformed bills, limb deformities and reduction/absence of eyes (Ohlendorf et al., 1998).

1.2 *Danio rerio* as a Model Organism – Experiment # 1

Danio rerio, commonly known as the zebrafish, has been accepted as a useful model organism for many areas of research. Native to the Himalayan regions, they commonly inhabit slow moving streams, canals, ponds or stagnant water bodies (Spence et al., 2007). Named for its resemblance to the zebra, *Danio rerio* have five uniform, horizontal blue stripes on the side of their body, which extend to the end of the caudal fin. They are currently being used to investigate a great deal of vertebrate biology including cardiophysiology, pharmacology, immunology, toxicology, developmental biology, genetics and many more areas. The zebrafish have a number of features that make them a model organism and there are numerous standardized protocols available to raise these fish in a controlled laboratory experimental setting (Westerfield, 1995; Brand et al., 2002). Zebrafish have a very short life cycle, reaching sexual maturity in less than three months and are capable of producing mass quantities of eggs in a single spawning event (Hill et al., 2005; Heiden et al., 2009). While rodents have only 5-10 offspring per pairing, zebrafish can produce upwards of 200-300 eggs in a single spawning event (Hill et al., 2005). In addition, the optical transparency of zebrafish embryos makes them easy to visualize and image organ developmental processes. This is also helpful for histochemical and morphological analysis (Hill et al., 2005; Berry et al., 2007; Vogt et al., 2009). Large-scale molecular information is also available as the entire zebrafish genome has been sequenced and annotated. Zebrafish share approximately 70 percent of protein-coding human genes, including roughly 84 percent of genes known to be associated with human diseases (Howe et al., 2013). This genetic similarity allows for great insight into the progression and development of human diseases. Normal heart

morphology and cardiac function have also been well documented *in vitro* as well as *in vivo* (Ho et al., 2002; Sun et al., 2008). In addition, electrophysiological studies have revealed that adult zebrafish have similar action potentials as humans, making them a suitable model for studying cardiac arrhythmias (Milan et al., 2006). Finally, zebrafish are small in size allowing for relative ease in handling and minimal space required for housing. For all the above reasons, zebrafish are an extremely useful model organism for research.

1.3 *Oncorhynchus mykiss* as a Model Organism – Experiment # 2

Oncorhynchus mykiss, commonly known as the rainbow trout, is a freshwater member of the Salmonidae family and are currently being used to investigate a great deal of vertebrate biology, including toxicology, pharmacology, carcinogenesis, disease ecology, genetics, immunology and many more areas. There are again numerous standardized protocols available to raise these fish in a controlled laboratory experimental setting and they have a number of features that make them a model organism. Extensive information has been collected for this species as it has long been cultivated for food and been considered as a sport fish. Their relatively large size compared to the zebrafish and other small model organisms allows for greater tissue harvesting and for non-terminal blood extraction (Thorgaard et al., 2002). Rainbow trout are widely distributed across North America, have great commercial importance and are known to be sensitive to the effects of environmental pollutants (Rand, 1995). They are closely related to the *Salmo* genera and are considered ideal in research as a proxy for economically and ecologically important species, such as Atlantic salmon (*Salmo salar*) (Thorgaard et al., 2002). Large-scale molecular information is also available through extensive characterization of their

genome over the years (Thorgaard et al., 2002). Moreover, Environment Canada and the United States Environmental Protection Agency (USEPA) currently uses the rainbow trout as a regulatory indicator for acute freshwater toxicity (Thorgaard et al., 2002) and they are therefore considered as an important bio-indicator of ecosystem health.

1.4 Endpoints and Biomarkers

Chronic exposure to contaminants can disturb several components of an organism's physiological processes. By studying these physiological changes, one can determine mechanisms of toxic action that lead to adverse outcomes on organismal health.

1.4.1 Cardiovascular implications

In contrast to the mammalian heart, the fish heart consists of only two chambers, a single atrium and a single ventricle. The closed circulatory system sees blood flowing from the sinus venosus, through the atrium and ventricle, then directed towards the gills for oxygenation via the bulbous arteriosus (Hu et al., 2001). Depending on their cardiovascular demands, different teleosts can have different ventricular shapes. Sedentary, less active organisms usually have tubular or saccular ventricles while more active teleosts, such as zebrafish and trout generally have pyramidal ventricles (Sanchez-Quintana et al., 1995). The distinctive ventricles have different organization of the myocardial fibres, playing a major role in the heart's oxygen supply. Pyramidal ventricles have a mixed myocardium, with multiple layers of compact tissue (compact layer) and a single inner layer known as the spongy (trabeculated) layer. This is in contrast to tubular and saccular ventricles consisting of only a single spongy layer. This allows pyramidal ventricles to receive oxygen from venous blood in the spongy layer, in addition to oxygenated blood from the compact layer via coronary arteries (Sanchez-Quintana et al.,

1995). This is compared to myocardium consisting of only trabeculated ventricles, where the only oxygen supply to the heart is by deoxygenated venous blood (Olson and Farrell, 2006).

Although it is known that chronic Se exposure can affect metabolic capacity, energy homeostasis and swimming performance in zebrafish (Janz et al., 2010; Thomas and Janz, 2011; Thomas et al., 2013), the underlying cardiovascular implications have yet to be explored. Cardiac activity is regulated in response to changes in oxygen supply and demand, and previous studies (Lemly, 1993; Scott and Sloman, 2004; Thomas and Janz, 2011) have shown that the oxygen consumption (MO_2) of fish exposed to Se were consistently greater than that of control fish. The measurements of MO_2 serve as an important indicator of metabolic capacity and aerobic performance of fish, and alterations to these values could be attributed to impaired aerobic performance (MacKinnon and Farrell, 1992). With an increase in oxygen demand, one would expect to see an increase in cardiac output, allowing the fish to receive sufficient amounts of blood, nutrients and oxygen. Previous exposure studies have however shown the opposite trend. Zebrafish exposed to beta-naphthoflavone (BNF) had an increase in oxygen consumption, a decrease in swim performance but a significant decrease in cardiac output, suggesting impaired cardiac function (Gerger et al., 2015).

A number of different parameters can be measured to quantify fish heart function and comparing these values to contaminant-exposed fish can help elucidate potential mechanisms of Se toxicity (Sun et al., 2008; Gerger et al., 2015; Nair et al., 2016). By investigating atrial versus ventricular contractile rates in fish hearts, we can look for evidence of electrical abnormalities through the atrioventricular (AV) node. A reliable

way to investigate this involves looking at the atrial to ventricular contraction ratio. In a normal healthy heart, the atrium sends a wave of electrical excitation to the ventricle, thus causing every atrial contraction to be followed by a ventricular contraction. This should therefore produce an AV contraction ratio close to 1, and any abnormalities could be indicative of conduction dysfunction. If the ratio is greater than 1, this could suggest toxicant-induced AV conduction blockade, potentially making the heart vulnerable to more lethal arrhythmias. As the AV node is known to be susceptible to disruptions caused by oxidative stress, the link between Se and the electrical activity in the heart is of great interest.

In the past, histological analysis on fixed zebrafish hearts provided morphological information, however gave little insight on specific functionality (Hu et al., 2001). Rudimentary zebrafish heart function can be evaluated only at the earliest developmental stages when the embryo or larvae is still transparent using microscopic visualization of the heart (Incardona et al., 2011; Corrales et al. 2014). However, assessing cardiac or vascular function in juvenile and adult fish requires more advanced techniques. At the cost of having to sacrifice the test subject, previous studies on rainbow trout and a variety of sunfish (*Lepomis*) species have used invasive methods to surgically implant probes to characterize blood flow (Cooke et al., 2010; Gamperl et al., 2012). While these methods were successful in providing information on blood velocity and cardiac output, a new technology known as high frequency ultrasound has proven to be an accurate, non-invasive method to evaluate fish cardiac function (Sun et al., 2008).

1.4.2 Ultrasound biomicroscopy

The use of zebrafish and rainbow trout as model organisms for cardiovascular studies continues to grow, and the technology surrounding it has dramatically improved over the past decade. As the zebrafish heart has structural similarity to the mammalian heart, it serves as an excellent model for experimental studies (Weinstein and Fishman, 1996). The optical transparency and external organ development in zebrafish embryos allow for great visualization in their early development. This however, is limited to juvenile zebrafish as the opacity of adults requires the use of high spatial resolution technology to visualize cardiovascular functions. High frequency ultrasound bio-microscopy has proven to be an accurate, non-invasive method to visualize fish cardiac function *in vivo*. This new technology allows for real-time detailed imaging of cardiac structures at a spatial resolution of 30μm and quantitative evaluation of the cardiovascular system by B-mode structural visualization and pulsed-wave Doppler blood flow measurements (Sun et al., 2008). This non-invasive technique provides the means to evaluate and quantify the function, valve movement and blood flow of major vessels, including the ventricle, atrium, sinus venosus and bulbus arteriosus. High-frequency ultrasound can also be used to assess the progression of cardiovascular pathologies such as diastolic dysfunction, vessel stenosis and fibrosis.

In order to evaluate overall cardiovascular function, numerous cardiac endpoints will be quantified in my thesis research. Among these include: heart rate, stroke volume, cardiac output, ejection fraction, Doppler ejection velocity, and ventricular and atrial contractile rate. These measurements will give insight into any cardiovascular effects Se exposure may affect.

1.4.3 Oxidative stress

As previously mentioned, the leading hypothesis surrounding the ability of Se to exert its toxic effects results from Se-induced oxidative stress. Selenium metabolism leads to the formation of redox reactive intermediates, which react with glutathione and generate toxic hydrogen peroxide, superoxide anions and hydroxyl radicals (Spallholz et al., 1994; Spallholz et al., 2001). When the production of these toxic reactive oxygen species (ROS) overwhelms the endogenous cellular antioxidant defense mechanisms, oxidative stress will occur. The damage caused by ROS can include lipid peroxidation, DNA oxidation, proteolysis and cellular apoptosis. In order to protect cellular macromolecules from ROS damage, organisms have developed numerous antioxidant defense mechanisms, however exposure to toxic contaminants can overwhelm their abilities leading to an oxidized environment, rather than the desired reduced state. For these reasons, antioxidant concentrations can be useful biomarkers of oxidative damage caused by contaminant exposure. Evaluating the concentrations of specific antioxidant enzymes such as glutathione peroxidase, glutathione-s-transferase, superoxide dismutase, and catalase can be functional biomarkers of ROS production. For example, previous studies investigating the influence of dietary Se on antioxidant status and oxidative stress-related parameters in tilapia (*Oreochromis niloticus)* and rainbow trout showed a significant decrease in the reduced to oxidized glutathione ratio (GSH:GSSG) and an increase in glutathione peroxidase activity with increased Se concentrations (Schlenk et al., 2003; Atencio et al., 2008). Furthermore, loaches (*Paramisgurnus dabryanus*) exposed to increasing concentrations of dietary Se showed a significant increase in superoxide dismutase, glutathione peroxidase and catalase activities (Hao et al., 2014). In addition, as the

cardiovascular system is known to be particularly susceptible to oxidative stress, investigating antioxidant mRNA abundance can be a useful biomarker of oxidative damage caused by contaminant exposure.

1.4.4 Triglyceride and glycogen Stores

Triglycerides and glycogen are the key forms of stored energy in fish (Dangé, 1986; Tocher, 2003; Thomas and Janz, 2011), and these energy stores are utilized for locomotion, growth, reproduction, and detoxification processes. Exposure to environmental contaminants has been shown to significantly alter triglyceride and glycogen concentrations in fish, and such effects may threaten the overall growth and survivability of fish (Dange, 1986; Levesque et al., 2002; Bennett and Janz, 2007). Specifically, fish collected from Se-contaminated sites and in controlled laboratory experiments have shown significant increases in both glycogen and triglycerides (Bennett and Janz, 2007; Wiseman et al., 2011a,b; Thomas and Janz, 2011). Fish survival is highly dependent on swimming ability. Food acquisition, predator avoidance, migration and mating all rely on adequate swimming performance and any impairment or dysfunction can have drastic effects on the individual, potentially leading to overall population and ecosystem effects (Scott and Sloman, 2004). Triglycerides are the principal lipids contained in fish energy reserves, and are primarily stored in the liver, muscle, visceral tissue and gonads (Jobling, 1994). These lipids provide an excellent source of energy for sustained and prolonged swimming by the aerobic mobilization of free fatty acids via triglyceride β-oxidation (Goertzen et al., 2011). Rats exposed to dietary Se-Met were reported to have elevated triglyceride synthesis by increased fatty acid synthase (FAS) activity (Mueller et al., 2008). Furthermore, zebrafish and rainbow trout exposed to

elevated dietary Se-Met exhibited increased triglyceride levels in liver and muscle tissue (Thomas and Janz, 2011; Wiseman et al., 2011b). With these increases in energy storage, one would assume the fish would be able to sustain longer physical exertion, allowing it to have a greater swimming performance. This however isn't the case as described by Thomas and Janz (2011) and McPhee and Janz (2014). Contrarily to what we would assume, these high exposure Se-Met fish with elevated energy stores showed almost a 50% reduction in swimming performance compared to the control (Thomas and Janz, 2011).

While sustained and prolonged swimming is primarily powered by slow oxidative red muscle fibers, fueled by triglycerides, swimming speed beyond 80% of their critical swimming speed (U_{crit}) in fish is powered by fast glycolytic white muscle fibers, which comes from the anaerobic catabolism of glycogen (Hammer, 1995; Moyes and West, 1995). Glycogen is an essential form of energy primarily located in the liver and muscle tissue, and functions as the main long-term energy store in fish (Jobling, 1993). The quick breakdown of intramuscular glycogen helps provide the fish with the necessary energy required for burst swimming (Webb, 1971; Hammer, 1995). Essential for its role in the physiological stress response, glycogen can be rapidly metabolized to supply energy for activities such as predator avoidance, territorial defense and actively searching for food (Webb, 1971; Hammer, 1995). Chronic exposure to environmentally relevant concentrations of dietary Se has been demonstrated to cause significantly increased glycogen storage in zebrafish and rainbow trout (Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013). Once again we would presume this to be beneficial, allowing the fish to have greater energy reserves to utilize in physically demanding times.

But as stated above, these highly exposed fish have significantly reduced swimming performance when compared to controls. The increase in energy storage coupled with the decrease in aerobic performance leads to the hypothesis that the fish are having difficulties accessing their reserves. These changes could also potentially be attributed to alterations in enzyme activities involved in energy metabolism. Enzymes of interest to investigate include lactate dehydrogenase, phosphofructokinase and citrate synthase; all of which have been shown to have altered enzymatic activities following pollutant exposures in various fish species (Almeida et al., 2001; Konradt and Braunbeck, 2001).

For the above reasons, the determination of triglyceride and glycogen concentrations can be considered as a useful tool in investigating the overall health and metabolic capacity of fish following exposure to toxicants.

1.4.5 Gene expression biomarkers

Gene expression measurements of enzymes involved in cardiovascular function and energy metabolism can help provide a mechanistic understanding of sublethal toxicities faced by organisms inhabiting Se-contaminated aquatic ecosystems. To help elucidate the potential mechanisms of metabolic dysfunction, aerobic metabolism enzymes such as citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD) could be investigated. HOAD is directly involved in the metabolism of triglycerides whereas CS is a key enzyme involved in the citric acid cycle. Previous studies have identified altered enzyme activities of both CS and HOAD after exposure to stressors (Rajotte and Couture 2002; Goertzen et al., 2011, 2012). These enzymes could therefore be considered to investigate the underlying cellular metabolic effects of dietary Se-Met in adult zebrafish.

Considering the potential for Se-induced cardiac dysfunction, markers of interest to be investigated include matrix metalloproteinases (MMP), serpin peptidase inhibitor, clad H1 (SERPINH1), and sarcoplasmic reticulum Ca-ATPase (SERCA). MMPs are a family of proteolytic enzymes with important cardiac and vascular remodeling properties and have been shown to be up-regulated in hypertensive patients (Liu et al., 2006; Niemirska et al., 2016). SERPINH is a stress inducible protein in the endoplasmic reticulum, plays a major role as a molecular chaperone, essential for the post-translational folding of fibril-forming collagens (Rocnik et al., 2002). Increases in SERPINH concentrations are associated with increases in extracellular matrix formation, which can lead to an increase in fibrotic diseases (Rocnik et al., 2002). Finally, SERCA is a major component in cardiac calcium cycling and reduced levels have been associated with diastolic dysfunction in mouse models (Lalli et al., 2001). Coupled with the results from the ultrasound biomicroscope, these molecular responses may aid in elucidating the underlying mechanisms of the Se toxicity.

1.4.5 Diabetes

The link between Se supplementation and the incidence of type 2 diabetes (T2D) has been the subject of debate for many years. While past literature indicates that Se deficiency may contribute to insulin resistance and the progression of T2D in mammals (Kljai and Runje, 2001), and with adequate Se supplementation, the risk of T2D is diminished (Park et al., 2012), many other studies have observed the opposite. Increasingly, both rodent and human studies suggest an excess of Se may similarly impair insulin sensitivity to produce a T2D-like state (Rasekh et al., 1991; McClung et al., 2004; Stranges et al., 2007; Bleys et al., 2007; Laclaustra et al., 2010; Wang et al., 2008;

Mueller et al., 2008; Mueller et al., 2009; Stranges et al., 2010, 2011; Labunsky et al., 2011; Zeng et al., 2012; Liu et al., 2012; Wang et al., 2014). Some reactive oxygen species are required for proper insulin signaling, but an imbalance caused by excess Se toward a more pro-oxidant oxidative stress state is suggested to mediate this change in insulin sensitivity, with selenoprotein P and glutathione peroxidase inactivation by ROS being key triggers (Steinbrenner, 2013; Wang et al., 2014). In animal models of human disease, excess dietary selenium has been shown to impair insulin-regulated carbohydrate and lipid metabolism through a mechanism involving reactive oxygen species to produce diabetes (Steinbrenner, 2013; Wang et al., 2014). Moreover, prolonged hyperglycemia is associated with increased circulating levels of the toxic glucose metabolite, methylglyoxal, in mammals (Desai et al., 2010; Adolphe et al., 2012; Kalapos, 2013). Methylglyoxal, in turn, is known to promote oxidative stress and is a precursor to advanced glycation end products responsible for development of diabetic complications such as cataracts (Desai et al., 2010; Kalapos, 2013). This observation agrees well with previous studies reporting cataracts in rats exposed to selenite (Shearer et al., 1983) or fish from a high Se environment at Belews Lake, North Carolina (Lemly, 2002). Selenium has been shown through confocal X-ray synchrotron-based fluorescent imaging to preferentially accumulate in the eye lens in zebrafish (Choudhury et al., 2015). In addition, adult zebrafish vision has been investigated using behavioural assays, and adults fed with Se-Met spiked diets exhibited reduced escape responses and their F1 progeny had smaller eyes and fewer positive responses in phototaxis, oculomotor and optokinetic response assays (Raine et al., 2016). Glutathione peroxidases (GPX1 and GPX3; both selenoproteins) found in the eye (Pirie, 1965) normally protect the lens from oxidative

damage (Flohe, 2005), but a previous study reported excess Se caused decreased anti-oxidants and increased oxidative damage in the eye (Combs and Combs, 1986).

In fish, even in the absence of overt diabetes, inadequate mobilization of energy stores could lead to impaired swimming and cardiovascular impairment. Whether a similar link exists between excess selenium, impaired energy mobilization, cardiotoxicity and development of impaired glucose control in fish is unclear.

1.5 Purpose of Research

The efficient incorporation of Se into the aquatic food web is a cause for concern as higher trophic levels, especially oviparous species, are highly susceptible to increased Se concentrations. Anthropogenic activities such as coal, phosphate and uranium mining, as well as fertilizer use, have greatly exacerbated the loading of Se into the aquatic environment. As Se is known to have a narrow margin between essentiality and toxicity, there is a need to further characterize the chronic physiological effects of supraphysiological dietary Se concentrations.

Therefore, the purpose of my research is to further investigate mechanisms of Se toxicity, particularly potential underlying cardiovascular implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met in both zebrafish and rainbow trout.

1.5.1 Objectives:

1. To elucidate the underlying cardiovascular and metabolic implications involved in Se-induced toxicities in adult zebrafish and juvenile rainbow trout
2. To investigate at the cellular level what genes are involved in this cardiac and metabolic dysfunction in adult zebrafish and juvenile rainbow trout

1.5.2 Hypotheses:

3.1 H_0: Exposure to elevated dietary Se-Met will have no effect on cardiovascular function in adult *Danio rerio* and yearling *Oncorhynchus mykiss*

3.2 H_0: Exposure to elevated dietary Se-Met will have no effect on energy homeostasis in adult *Danio rerio* and yearling *Oncorhynchus mykiss*

3.3 $H_{0:}$ Exposure to elevated dietary Se-Met will show no effect in relation to the expression of genes of interest in adult *Danio rerio* and yearling *Oncorhynchus mykiss*

CHAPTER 2

2.0 CARDIOVASCULAR AND METABOLIC EFFECTS OF DIETARY SELENOMETHIONINE EXPOSURE IN ADULT ZEBRAFISH

Preface

The research in this chapter was designed to address the underlying cardiovascular and metabolic implications of dietary selenomethionine exposure in adult zebrafish.

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Pettem, C.M., Weber, L.P., and Janz, D.J., (2017). Cardiac and metabolic effects of dietary selenomethionine exposure in adult zebrafish. *Tox. Sci.* **159**, 449-460.

The author contributions to chapter 2 of this thesis were as follows:

Connor Pettem (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

Lynn Weber (University of Saskatchewan) helped design the study, provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

2.1 Abstract

Selenium (Se) is an essential micronutrient involved in important metabolic functions for all vertebrate species. As Se is reported to have a narrow margin between essentiality and toxicity, there is growing concern surrounding the adverse effects of elevated Se exposure caused by anthropogenic activities. Recent studies have reported that elevated dietary exposure of fish to selenomethionine (Se-Met) can alter aerobic metabolic capacity, energetics and swimming performance. This study aims to further investigate mechanisms of sublethal Se-Met toxicity, particularly potential underlying cardiovascular implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met in adult zebrafish (*Danio rerio*). Adult zebrafish were fed either control food (1.1 μg Se/g dry mass [d.m.]) or Se-Met spiked food (10.3 or 28.8 μg Se/g d.m.) for 90 d at 5% body weight per day. Following exposure, ultrahigh resolution B-mode and Doppler ultrasound was used to characterize cardiac function. Chronic dietary exposure to elevated Se-Met significantly reduced ventricular contractile rate, stroke volume, and cardiac output. Exposure to Se-Met significantly decreased mRNA expression of methionine adenosyltransferase 1 alpha and glutathione-S-transferase pi class in liver, and a key cardiac remodelling enzyme, matrix metalloproteinase 2, in adult zebrafish heart. Se-Met significantly increased echodensity at the junction between atrium and ventricle, and these results combined with increased matrix metalloproteinase 2 expression are consistent with cardiac remodelling and fibrosis. The results of this study suggest that chronic exposure to dietary Se-Met can negatively impact cardiac function, and such physiological consequences could reduce the aerobic capacity and survivability of fish.

2.2 Introduction

Selenium (Se) is an essential micronutrient involved in important metabolic and physiological functions in all vertebrate species. Selenium is known to have a narrow margin between essentiality and toxicity, particularly in oviparous vertebrates such as fishes (Janz et al., 2010). Under normal physiological conditions, fish require dietary concentrations of 0.1 to 0.5 μg Se/g dry mass (d.m.) to maintain normal selenoprotein function, however toxicity has been shown to occur at concentrations exceeding 3 μg/g d.m (Janz, 2012). Selenium is commonly found in areas of sedimentary rock depositions, in particular phosphate, black shale and coal deposits (Presser et al., 2004; Maher et al., 2010), and naturally enters aquatic environments in relatively low concentrations (Maher et al., 2010). Petroleum refineries, mining and agricultural industries, as well as coal-fired power plants are all areas of economic importance that can exacerbate the loading of Se into the environment by manufacturing waste by-product generation (Lemly, 2004; Maher et al., 2010; Janz, 2012). Inputs of inorganic Se are taken up by primary producers and microorganisms, then biotransformed into organoselenoproteins, predominantly selenomethionine (Se-Met) and selenocysteine (Se-Cys) (Fan et al., 2002). Trophic transfer of organic Se amongst primary consumers, secondary consumers and finally higher order consumers including birds, fish, and humans occurs primarily through dietary pathways. Se-Met is the principal dietary source of Se available to fish (Fan et al., 2002), and due to the structural similarity between Se-Met and the amino acid methionine, Se-Met can avoid biotransformation and be directly integrated into any methionine-containing protein in a non-specific, dose-dependent manner (Behne et al., 1991). Due to its biotransformation to selenoxides, such as methylselenol, excessive Se-

Met uptake has been shown to cause oxidative stress *in vitro* (Palace et al., 2004; Spallholz et al., 2004). While it is generally agreed that protein dysfunction and oxidative stress are the main mechanisms underlying Se toxicity, fish exposed to excess Se in the form of selenite or Se-Met have also shown impaired cellular methylation (Ma et al., 2012; Thomas et al., 2013).

Using zebrafish (*Danio rerio*) as a model test organism, elevated exposure to Se-Met have previously been reported to alter aerobic metabolic capacity, respiration, energy homeostasis, swimming performance and cause a greater incidence of early life stage deformities and mortalities (Tashjian et al., 2006; Thomas and Janz, 2011; Thomas et al., 2013; Arnold et al., 2016). However, there is a lack of research investigating the potential role of cardiovascular dysfunction associated with such responses in fishes exposed to elevated dietary Se exposure. Cardiac activity is regulated in response to changes in oxygen supply and demand, and previous studies (Scott and Sloman, 2004; Thomas and Janz, 2011; Thomas et al., 2013) have shown that the oxygen consumption (MO_2) of fishes exposed to Se were consistently greater than that of control fish. Advances in high frequency ultrasound bio-microscopy have proven to be an accurate, non-invasive approach to visualize fish cardiac function *in vivo* (Gerger et al., 2015; Gerger and Weber, 2015). Natural and anthropogenic stressors have been shown to alter both metabolic energy expenditure and storage, as well as impair the physiological stress response in fishes (Mommsen et al., 1999; Thomas and Janz, 2011; Wiseman et al., 2011; Thomas et al., 2013). Thus, impairment of these key physiological responses may cause a downstream cascade effect on important physiological functions, including the cardiovascular system.

Glycogen and triglycerides are the two main forms of energy stored in fish and are predominantly used in burst and prolonged swimming, respectively (Hammer, 1995; Moyes and West, 1995). Fish collected from Se-contaminated sites, as well as those fed a range of excess Se-Met in controlled laboratory settings exhibited elevated levels of stored glycogen and triglycerides (Bennett and Janz, 2007; Wiseman et al., 2011; Thomas et al., 2013). Inadequate mobilization of these energy stores could lead to impaired swimming, but also cardiovascular disease.

The overall goal of this study was to further investigate sublethal mechanisms of Se toxicity in adult zebrafish, particularly potential underlying cardiovascular and metabolic implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met. Cardiac function was assessed *in vivo* using ultrahigh resolution ultrasonography, followed by quantification of energy stores in muscle and mRNA transcript abundance of selected genes involved in aerobic metabolism, cardiac function, oxidative stress and cellular methylation.

2.3 Materials and Methods

2.3.1 Test compound

Seleno-L-methionine (> 98% purity) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.3.2 Test species

Adult zebrafish were purchased from a local supplier and housed in an environmental chamber with controlled temperature (28.0 ± 1.0°C) and photoperiod (14 h light: 10 h dark). Zebrafish were randomly selected from an existing colony and placed into three 40 L glass aquaria (32 fish/tank) with continuous aeration and particulate, charcoal, and

ammonia biofilters. Fish were then acclimated for 2 weeks to these conditions and fed Nutrafin basic flake food (Hagen Inc., Montreal, QC, Canada) twice daily prior to the beginning of the dietary exposure. Experiments were conducted according to procedures approved by the University of Saskatchewan Animal Care and Use committee according to the Canadian Council on Animal Care guidelines.

2.3.3 Diet preparation

Nominal concentrations (10 and 30 µg/g d.m.) of Se in the form of Se-Met were dissolved in nanopure deionized distilled water, added to ground Nutrafin flake food, and mixed thoroughly for approximately 20 min. The control diet was made by replicating the above process with an equal volume of water, without the addition of Se-Met. Water was removed from all the diets using a freeze dryer (Dura-DryTM MP, FTS Systems, Stone Ridge, NY, USA). Freeze dried diets were then crushed into flakes with a mortar and pestle, and stored at -20°C for the remainder of the experiment. Sub-samples of each diet were collected for total Se analysis using inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada).

2.3.4 Feeding exposure

Each treatment group consisting of 32 fish was fed twice daily (5% body weight/day ration) with either control or Se-Met spiked diets for 90 d. Throughout the exposure, fish were allowed to feed for approximately 2 h, after which all excess food was siphoned, and 75% water renewals were performed in each aquarium every 2 d. Following the 90 d exposure, n = 3 fish from each treatment were randomly selected, euthanized with an overdose of tricaine methanesulfonate (MS 222) (1g/L) followed by spinal severance and

stored at -80°C for subsequent whole body Se analysis. In addition, n = 12 fish from each treatment were randomly selected for cardiac ultrasonography as described in section 2.6. Following ultrasonography, fish were euthanized as above and heart, liver and muscle were collected. All remaining fish were euthanized and organs collected. Heart and liver samples were placed in RNAlater (Thermo Fisher Scientific Inc., Waltham, MA, USA) prior to -80°C storage and were used for mRNA analysis of genes of interest using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Muscle samples were placed in -80°C storage and used for quantification of metabolic energy stores (glycogen and triglycerides). Prior to dissection and storage, fish morphometrics (total length, body weight and condition factor) were determined for all fish.

2.3.5 Total Se analysis

Food and whole body zebrafish samples were lyophilized using a freeze dryer and homogenized using a tissue homogenizer (BioSpec Products, Bartlesville, OK). Moisture content of whole body zebrafish was 72.5 ± 1.7%. 100 mg aliquots were cold digested in Teflon vials with 5 ml of ultra-pure nitric acid and 1.5 ml of hydrogen peroxide. Upon digestion, samples were evaporated on a hot plate (<75°C), reconstituted in 5 ml of 2% ultrapure nitric acid, and stored at 4°C until ICP-MS analysis could be performed to determine total Se concentrations. A method detection limit of 0.7 µg Se/g d.m. was determined using method blanks, and the recovery of Se (100.8 ± 6.3%) was determined using a certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada)

2.3.6 Cardiovascular endpoint evaluation

In vivo cardiac imaging was performed using non-invasive ultrahigh frequency ultrasonography in order to quantify cardiovascular function. This technique was adapted from a rodent model to zebrafish, using a VEVO 3100 high frequency ultrasound (VisualSonics, Markham, ON, Canada) capable of B-mode imaging and pulse-wave Doppler (VisualSonics, 2008) to quantify cardiac function according to methods previously published by our laboratory (Gerger et al., 2015; Gerger and Weber, 2015). Prior to ultrasonography, fish were placed ventral side up in a small foam holder submerged in a container with continuous flow of aerated water under light anesthesia (15 mg/l) of Aquacalm (metomidate hydrochloride). Temperature was maintained at normal zebrafish housing temperature of 28 ± 0.7˚C using a heated recirculating water bath. A handheld transducer was then used to produce acoustic pulses up to 75 MHz toward the body cavity of zebrafish, producing real-time *in vivo* short-axis and long-axis imaging at a resolution of 30μm. B-mode images were collected and used to determine outer ventricular volume at systole and diastole (minimum of 3 images for each view at each stage of the cardiac cycle averaged per fish) using Simpson's rule of discs (Mercier et al., 1982; VisualSonics, 2008). Briefly, three different short axis views of the ventricle were used to calculate circular area (A_1, A_2, A_3). The ventricular length (l) from a long axis view was then measured and divided by the total number of short axis views (3) to give the height (h=1/3) of each ventricular disk and used to calculate end systolic (ESV) and diastolic volumes (EDV) using the Simpson's rule equation:

$$V = (A1+A2)h + ((A3h)/2) + (\pi/6(h^3)) \qquad (1) \qquad \text{(Mercier et al., 1982)}$$

End systolic volume was then subtracted from end diastolic volume to give ventricular stroke volume (V_S). All volume (EDV, ESV, V_S) and cardiac output data were also corrected for individual fish body weight.

$$V_S = \text{EDV} - \text{ESV} \qquad (2)$$

Ventricular and atrial contractile rates were calculated by counting individual beats in 5 second B-mode ultrasound video loops, then converting to beats per minute (BPM). Cardiac output (Q) was then calculated using ventricular contractile rate (f_H) and (V_S):

$$Q = f_H \text{ X } V_S \qquad (3)$$

In addition, in order to evaluate efficiency of atrial excitation to travel through the atrioventricular (AV) node to excite the ventricles, atrial contractile rate was divided by ventricular contractile rate to calculate the AV ratio. In ideal physiological conditions, this value should be equal to 1.0:

$$\text{AV Ratio} = \text{Atrial } f_H \text{ / Ventricular } f_H \qquad (4)$$

Finally, because B-mode imaging in preliminary experiments suggested that Se-Met may have altered echodensity of the zebrafish heart (tissues showing greater white values using B-mode imaging), Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD, USA) was used to quantify integrated optical echodensity surrounding the AV valve. This altered echodensity is indication of either increased calcification, such as would be the case for bone, or increased levels of fibrotic or calcified soft tissue, as is the case in cardiac tissue (DiBello et al., 1995). For these analyses, a minimum of 3 images per fish were analyzed in the same long-axis view at end-diastole after calibration of gray-scale from 0-100% density (complete black or echotransparent to complete white or echo-

opaque), with perimeters around areas showing greater white (echodense) areas hand-drawn, then integrated optical density calculated.

2.3.7 Determination of triglyceride and glycogen stores

Concentrations of muscle triglycerides were determined upon completion of the 90 d exposure using a kit prepared by Sigma-Aldrich (Oakville, ON, Canada) based on a lipase and glycerol kinase colorimetric assay, which has been previously validated for measuring triglycerides in whole fish homogenates in our laboratory (Weber et al., 2003; Bennett and Janz, 2007). Standard curves were prepared using a glycerol solution.

Concentrations of muscle glycogen were analyzed upon completion of the 90 d exposure using an amylase and glucose oxidase-based colorimetric assay, which has been validated in our lab (Weber et al., 2008). Reagents for the assay were purchased from Sigma-Aldrich. Standard curves were prepared using purified Type IX bovine liver glycogen.

2.3.8 Gene expression analysis

Expression of mRNA transcript abundance of genes coding for proteins of interest were quantified by use of qRT-PCR. Total RNA was extracted from muscle using RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON, Canada) and from liver and heart using the RNeasy Plus Mini Kit (Qiagen). Purified total RNA was then quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purified RNA samples were stored at −80 ºC until synthesis of cDNA. First-strand cDNA synthesis was performed using a QuantiTect® Reverse Transcription Kit (Qiagen) from 1 μg total RNA. The cDNA samples were stored at −20ºC until further analysis.

qRT-PCR was performed in 96-well plates by use of an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of gene specific primers were designed against target genes by use of PrimerBlast software (NCBI Biosystems, Bethesda, MD, USA). A separate 45 µl PCR reaction mixture consisting of Power SYBR Green master mix (Applied Biosystems), cDNA, gene specific primers, and nuclease free water was prepared for each cDNA sample and primer pair. A final reaction volume of 20 µl was transferred to each well and reactions were performed in duplicate, with an n = 10 in liver and muscle, and n = 5 pooled hearts (3 hearts/sample) per treatment. The PCR reaction mixture was denatured at 95ºC for 10 min before the first PCR cycle. The thermal cycle profile was performed as follows: denature for 10 s at 95ºC and extension for 1 min at 60ºC for a total of 40 PCR cycles. Optimal PCR conditions were established by determining the efficiency of each PCR assay with a standard curve of serially diluted cDNA standards. Target gene mRNA abundance was quantified by normalizing to the expression of elongation factor 1α (EF 1α) according to the Mean Normalized Expression (MNE) method of Simon (2003).

2.3.9 Statistical analysis

Data were tested for normality using the Shapiro-Wilk test and homogeneity of variance with the Levene test using SYSTAT 11 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post hoc test was used to determine significant differences among Se-Met treatments from the control group. All statistical tests used an alpha value of 0.05 to be considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

2.4 Results

2.4.1 Selenium analysis

Total Se concentrations in food and whole body zebrafish are shown in Table 2.1. Total Se concentration in the control diet was 1.1 ± 0.04 µg Se/g d.m. and in the Se-Met spiked food (nominal concentrations 10 and 30 µg Se/g d.m.) were 10.3 ± 0.05 and 28.8 ± 0.15 µg Se/g d.m. respectively, which were both significantly greater than the control diet ($p < 0.05$, Table 2.2). Whole body Se concentrations in zebrafish fed 1.1, 10.3 and 28.8 µg Se/g d.m. were 0.71 ± 0.16, 3.26 ± 0.30 and 9.19 ± 0.32 µg Se/g d.m., respectively, and both Se-Met exposed groups were significantly greater than control fish ($p < 0.05$, Table 2.1).

Table 2.1. Total Se concentrations in diets and whole zebrafish. Fish were fed either a control or selenomethionine-spiked diet for 90 d. Data are mean ± S.E.M. of n = 3 samples.

Nominal Diet [Se] (µg/g dry mass)	Dietary [Se] (µg/g dry mass)	Whole Body [Se] (µg/g dry mass)
Control	1.1 ± 0.04	0.71 ± 0.16
10 µg/g	10.3 ± 0.05*	3.26 ± 0.30*
30 µg/g	28.8 ± 0.15*	9.19 ± 0.32*

*, Significantly different from the control group using one-way ANOVA followed by Fisher LSD post-hoc test ($p < 0.05$).

Table 2.2. Mortality, total length, body weight and condition factor in adult zebrafish fed different concentrations of selenomethionine for 90 d. Cumulative mortalities were

calculated throughout 90 d exposure and morphometrics were determined on day 90. Data are mean ± S.E.M. of n = 15-16 fish

Dietary [Se] (µg/g dry mass)	Mortality (%)	Total Length (mm)	Body Weight (g)	Condition Factor
1.1	6.5	45.3 ± 0.7	1.12 ± 0.10	1.19 ± 0.08
10.3	15.6	43.8 ± 0.8	1.04 ± 0.08	1.21 ± 0.06
28.8	9.4	43.1 ± 0.6	1.00 ± 0.08	1.20 ± 0.07

Condition Factor = 100(body weight/total length^3)

2.4.2 Fish mortalities and morphometrics

Mortalities in control, 10.3 and 28.8 µg Se/g d.m. fed fish groups after 90 d were not significantly different among groups, being 6.5%, 15.6%, and 9.4%, respectively (Table 2.2). Total fish length in control and Se-Met spiked diets ranged from 3.7 to 4.9 cm and body mass of fish ranged from 0.7 to 1.9 g (Table 2.2). Condition factor (100×body weight/length^3) for control and Se-Met exposed fish ranged from 1.19 to 1.21 (Table 2.2). There were no statistically significant differences in any of the morphometric measures among exposed and control fish.

2.4.3 Cardiovascular function

Dietary Se-Met exposure had a pronounced effect on adult zebrafish cardiac function. Representative B-mode long axis view and colour Doppler flow sonograms as seen in Figure 2.1 were used to determine all cardiovascular endpoints. Although no significance was seen in end systolic volume between treatments, end diastolic volume was significantly decreased in fish fed 28.8 µg Se/g d.m. (2.9 ± 0.3 µl/g) when compared to control (4.0 ± 0.3 µl) (Figure 2.2 A, B). Ventricular contractile rate was significantly decreased ($p < 0.05$) in fish fed 28.8 µg Se/g d.m. (122.6 ± 5.1 BPM) compared to control

(140.3 ± 4.2 BPM) (Figure 2.2 C), while atrial contractile rate (data not shown) and ratio of atrial to ventricular contractile rates (AV ratio; Figure 2.2 D) both remained unchanged. Stroke volume was significantly decreased ($p < 0.05$) in fish from the highest exposure group compared to the control (0.97 ± 0.08 and 1.36 ± 0.15 µl/g, respectively) (Figure 2.2 E). Fish from the high Se-Met exposure group also showed a significant decrease ($p < 0.05$) in cardiac output (127.5 ± 9.6 µl/min/g) when compared to the control group (180.6 ± 18.4 µl/min/g) (Figure 2.2 F).

Representative sonograms are shown for control (Figure 2.3 A) and 28.8 µg Se/g d.m (Figure 2.3 B) groups in long-axis B-mode, showing an increase in echodensity (increased whiteness) at the junction between the atrium and the ventricle. Higher echodensity in cardiac tissue is associated with increased fibrosis or soft tissue calcification as has been previously reported in human diabetic hearts (Dibello et al., 1995). Quantification of the echodensity using integrated area of echodensity at the junction between the atrium and ventricle, surrounding the AV valve, showed significantly greater ($p < 0.001$) values in fish from the 10.3 and 28.8 µg Se/g d.m. treatments compared to the control (19.2 ± 1.4, 18.9 ± 1.5, and 12.3 ± 0.7, respectively) (Figure 2.3 C). A significant decrease in mRNA abundance in heart of the cardiovascular remodeling protein matrix metalloproteinase 2 (MMP2) was observed in the 28.8 µg Se/g d.m. group when compared to the control ($p < 0.05$; Figure 2.3 D). The velocity of blood flowing through the AV valve during active atrial ejection was quantitated using pulsed wave Doppler and a representative Doppler sonogram is shown (Figure 2.3 E). Quantitative analyses of the pulsed wave Doppler sonograms showed that velocity of the blood travelling through the AV valve during active atrial ejection was significantly

lower (150.7 ± 8.8 mm/s) in the 28.8 µg Se/g d.m. group compared to control (209.6 ± 11.7 mm/s) (Figure 2.3 F).

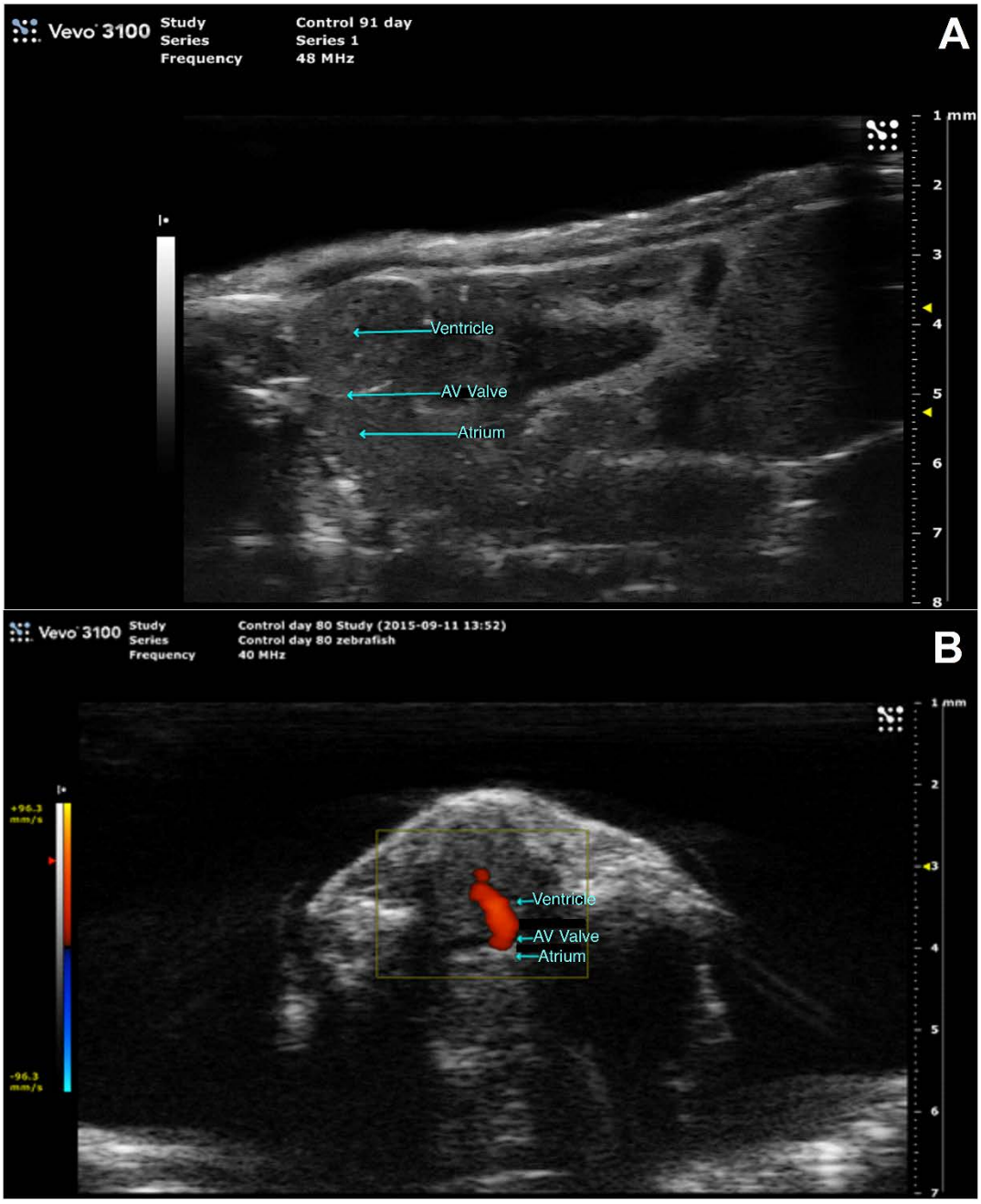

Figure 2.1. Representative long-axis brightness mode (B-mode) and colour flow Doppler short-axis views of the adult zebrafish heart. Anesthetized control zebrafish are imaged ventral side up, with B-mode showing general cardiac structures indicated by labelled arrows in panel A (AV = atrioventricular valve; V = ventricle; A = atrium). Blood flow direction and velocity are indicated by colour in panel B, with red indicating a slow to

moderate speed with unidirectional flow from atrium to ventricle through the atrioventricular valve using colour flow Doppler mode.

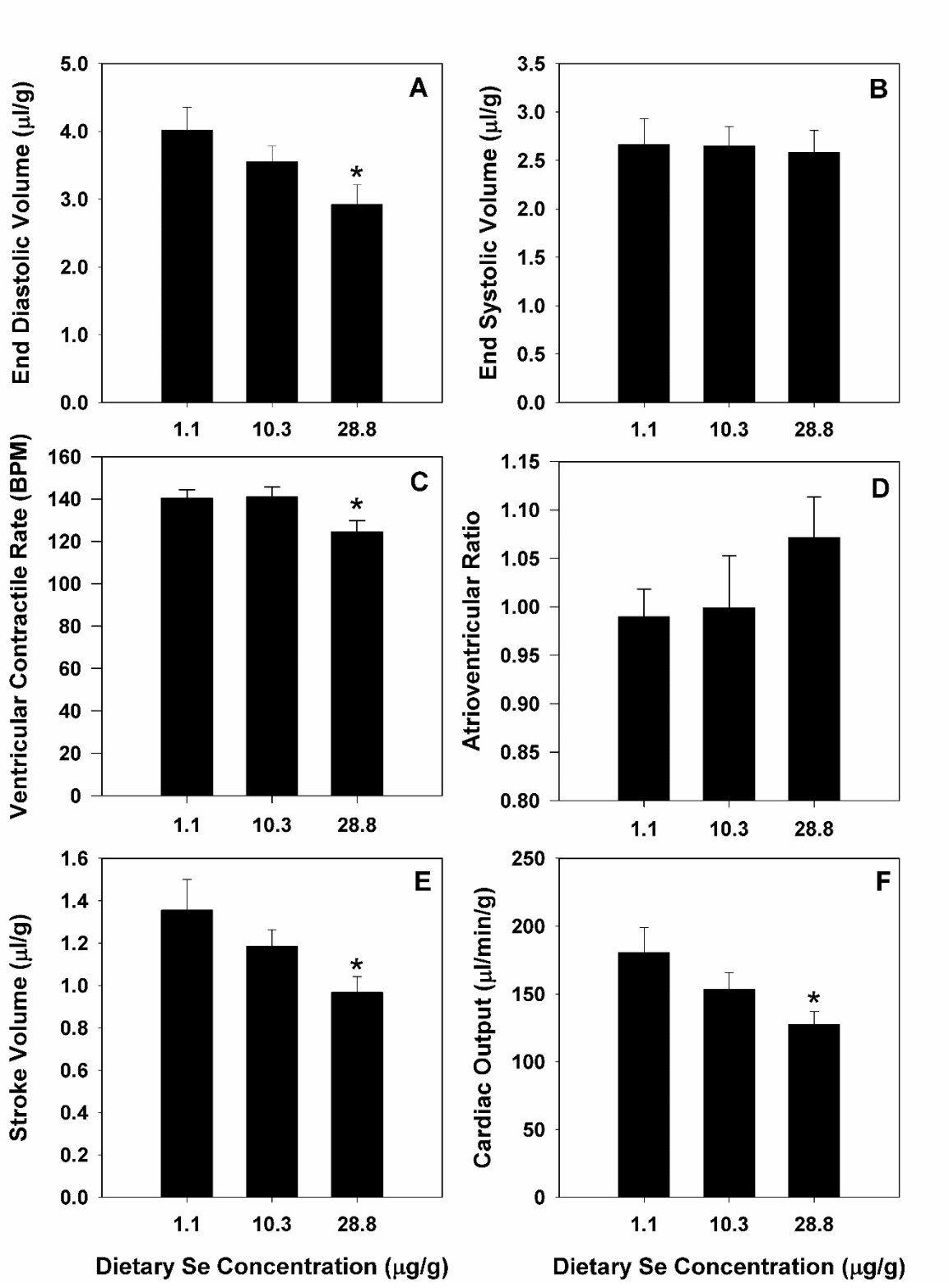

Figure 2.2. Quantitative analyses using cardiac ultrasound in zebrafish fed increasing levels of selenomethionine. **A**, End diastolic volume, **B**, end systolic volume, **C**,

ventricular contractile rate (beats per minute; BPM), **D**, atrioventricular ratio, **E**, stroke volume, and **F**, cardiac output of adult zebrafish fed control (1.1 µg Se/g d.m.) or selenomethionine spiked diets (10.3 and 28.8 µg Se/g d.m.) for 90 d. Data are expressed as mean ± S.E.M of n = 12 fish/group. *Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

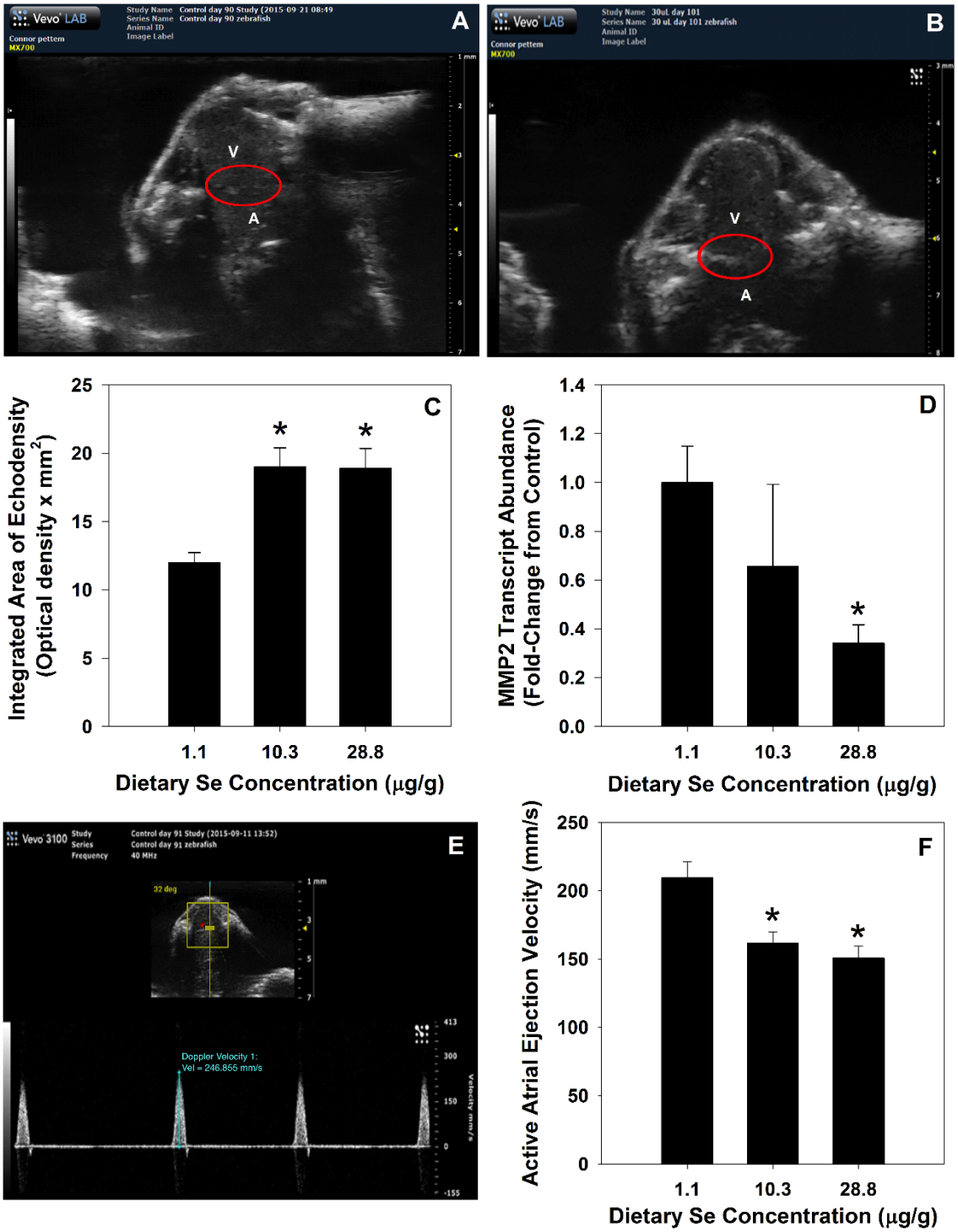

Figure 2.3. Representative B-mode sonograms in zebrafish from **A**, control group and **B**, 28.8 µg Se/g d.m group showing an increase in echodensity (white) at the junction between the atrium and the ventricle surround the atrioventricular (AV) valve (V =

ventricle; A = atrium). Higher echodensity is associated with increased fibrosis or soft tissue calcification. Gain (contrast) settings were equal between frames and treatments. Quantitation of the echodense areas using integrated optical density is shown in **C**. Data are mean ± S.E.M of n = 12-16 fish/group. mRNA abundance of the gene associated with cardiac remodelling is shown in **D**, matrix metalloproteinase 2 (MMP2) in heart. Data are mean ± S.E.M of n = 10 fish/group. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$). **E**, representative pulsed wave Doppler at AV valve, **F**, active atrial ejection velocity, measured as velocity of blood flow through the atrioventricular valve. Data are mean ± S.E.M of n = 12 fish/group. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

2.4.4 Energy storage and metabolic enzyme mRNA expression

Skeletal muscle and liver are the two tissues where glycogen and triglycerides are most abundantly used as metabolic stores in fish. These tissues are both highly metabolically active and were examined further for bioenergetic status. Due to limited organ size, only skeletal muscle energy stores were quantified. Muscle glycogen concentrations in fish fed 10.3 and 28.8 µg Se/g d.m. (4.4 ± 0.6 and 5.3 ± 0.8 mg/g, respectively) were significantly greater ($p < 0.05$) compared to control fish (1.8 ± 0.3 mg/g) (Figure 2.4 A). Muscle triglyceride concentrations in fish fed 1.1, 10.3 and 28.8 µg Se/g d.m. were 2.2 ± 0.1, 2.2 ± 0.2 and 2.6 ± 0.3 mg/g and no significant differences were observed among treatment groups (Figure 2.4 B). Genes associated with energy

homeostasis (β-hydroxyacyl coenzyme A dehydrogenase [HOAD] and citrate synthase [CS]), were quantitated in liver and skeletal muscle using RT-qPCR. A significant increase in liver, but not muscle, CS transcript abundance was observed in the 28.8 μg Se/g d.m. exposure group when compared to the control ($p < 0.05$, Figure 2.4 C). mRNA transcript abundance of HOAD was significantly reduced in both muscle and liver in the 10.3 and 28.8 μg Se/g d.m. exposed groups when compared to the control ($p < 0.05$; Figure 2.4 D).

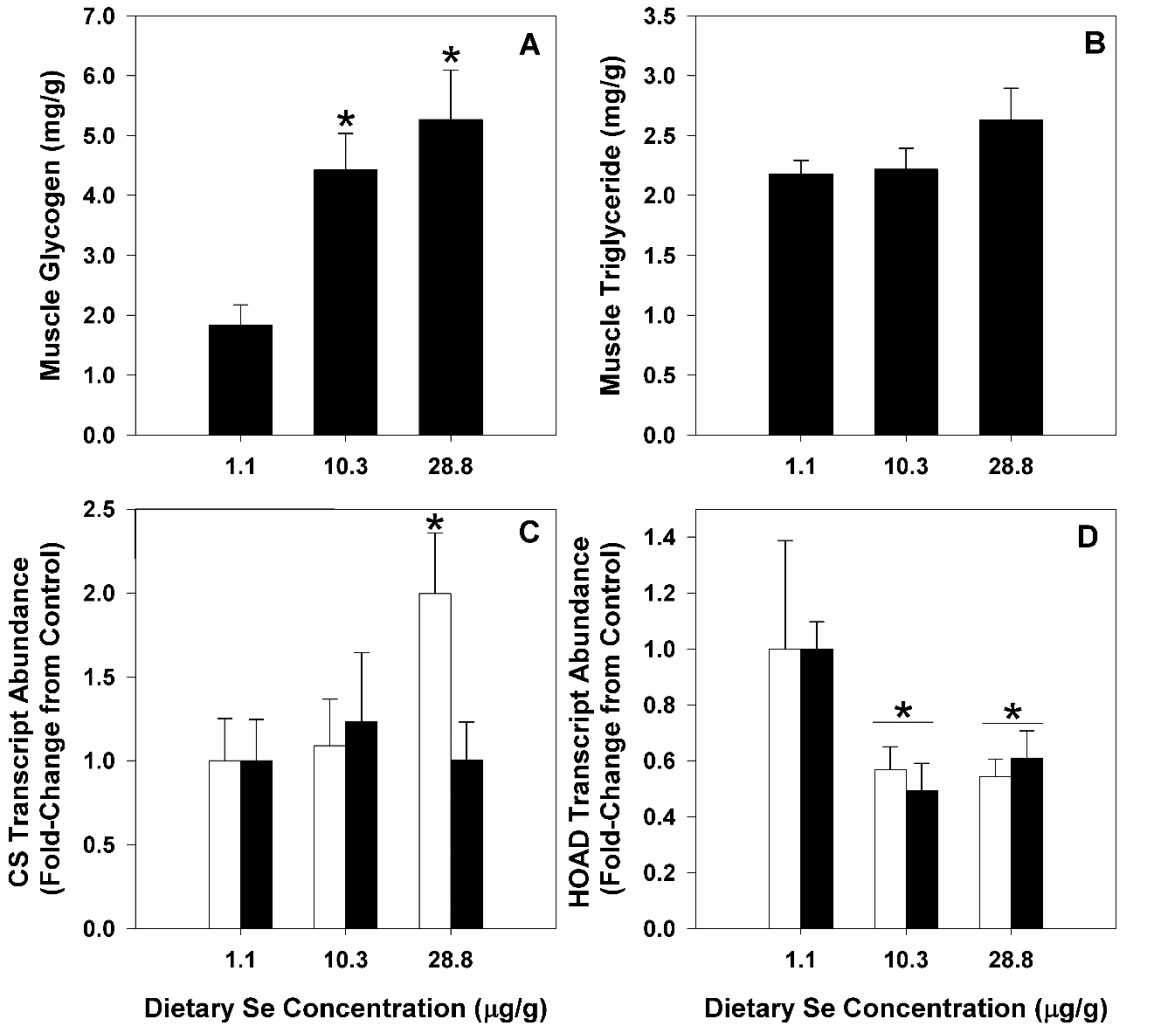

Figure 2.4. A, skeletal muscle glycogen concentrations and **B**, muscle triglyceride concentrations. Data are mean ± S.E.M of n = 12 fish/group. mRNA abundance of **C**, citrate synthase (CS) and **D,** β-hydroxyacyl coenzyme A dehydrogenase (HOAD) in liver (open bars) and muscle (closed bars) of adult zebrafish fed control (1.1 μg Se/g d.m.) or selenomethionine spiked diets (10.3 and 28.8 μg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. Data are mean ± S.E.M of n = 10 fish/group. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

2.4.5 Gene expression of Se-associated and antioxidant enzymes

Hepatic mRNA transcript abundances of cellular methylation reactions (methionine adenosyltransferase 1 alpha [MAT1A]), and Se transport (selenoprotein P [SEPP1a]) were determined. SEPP1a is synthesized in the liver and acts as the primary Se transport protein, and mRNA transcript abundance in liver increased in a dose dependent manner, with significantly greater expression observed in the 28.8 μg Se/g d.m. treatment compared to control ($p < 0.05$, Figure 2.5 A). A significant decrease in liver MAT1A mRNA transcript abundance was observed in both 10.3 and 28.8 μg Se/g d.m. treatment groups compared to the control (Figure 2.5 B). Gene expression of selected antioxidant enzymes (glutathione peroxidase 1A [GPX1A] and glutathione-S-transferase pi class [GST-pi]) were determined using RT-qPCR. A significant decrease in GST-pi mRNA abundance in heart was observed in the 10.3 μg Se/g d.m. group ($p < 0.05$; Figure 2.6 A). There were no significant differences in GPX1A mRNA abundances among treatments for liver and heart, although there was a trend for a dose-dependent increase in liver and decrease in heart (Figure 2.6 B).

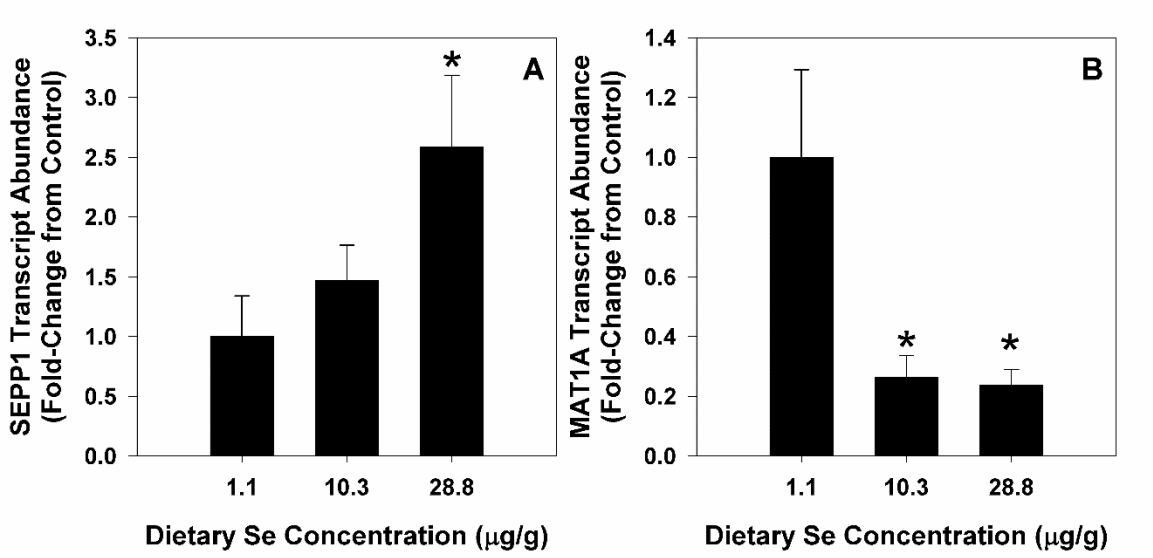

Figure 2.5. mRNA abundance of **A**, selenoprotein P (SEPP1a) and **B**, methionine adenosyltransferase 1 alpha (MAT1A) in liver of adult zebrafish fed control (1.1 μg Se/g d.m.) or selenomethionine spiked diets (10.3 and 28.8 μg Se/g d.m.) for 90 d. Transcript abundance was determined by quantitative real-time PCR. Data are mean ± S.E.M of n = 10 fish/group. *, Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

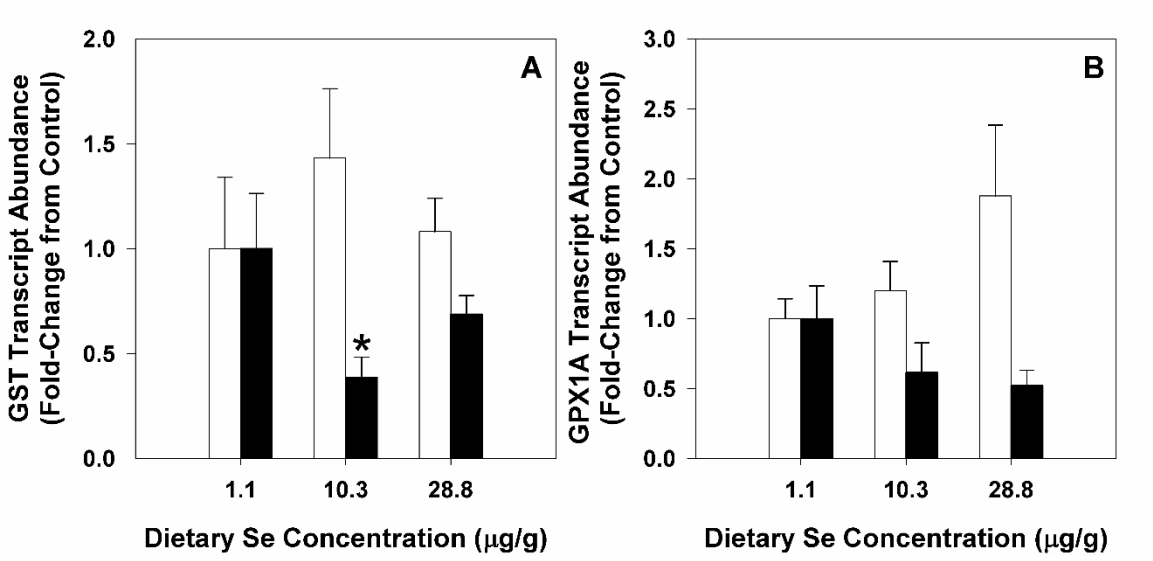

Figure 2.6. mRNA abundance of **A**, glutathione-s-transferase pi class (GST-pi) and **B**, glutathione peroxidase 1a (GPX1A) in liver (open bars) and heart (closed bars) of adult zebrafish fed control (1.1 μg Se/g d.m.) or selenomethionine spiked diets (10.3 and 28.8 μg Se/g d.m.) for 90 d. Data are mean ± S.E.M of n = 10 liver, and n = 5 pooled hearts (3 hearts/sample) per group. Transcript abundance was determined by quantitative real-time PCR. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

2.5 Discussion

To our knowledge, the present study was the first to investigate cardiovascular effects of chronic dietary Se exposure in adult fish. The most notable finding of this study was that environmentally relevant Se-Met exposure significantly impaired overall cardiac function in adult zebrafish. These effects included reduced stroke volume (the volume of blood pumped in each individual cardiac contraction), decreased ventricular contractile rate, and most notably, a decrease in cardiac output (the volume of blood pumped per minute). This would result in insufficient blood being ejected, thus reducing the amount of oxygen and nutrients being delivered to tissues (Olson and Farrell, 2006), causing further physiological impairment. Previous studies exposed zebrafish to similar dietary Se-Met concentrations and observed significant increases in oxygen consumption (MO_2), and reduced swim performance (U_{crit}), which were interpreted as reduced aerobic capacity (Thomas and Janz, 2011; Thomas et al., 2013). Cardiac activity is normally regulated in response to changes in oxygen supply and demand. Thus, greater cardiac output would be predicted in the elevated Se-Met exposed zebrafish based on higher oxygen demand, whereas we instead observed reduced cardiac output in the 28.8 µg Se/g d.m. treatment.

The underlying mechanisms for the observed cardiotoxicity are not clear, however two hypotheses can be proposed. As mentioned earlier, excessive Se-Met uptake is closely linked to toxicity due to its transformation to toxic reactive oxygen species (Spallholz et al., 2004). In a normal healthy heart, each atrial beat should send electrical excitation to the ventricle to generate a corresponding excitation/contraction. Thus, the ratio of atrial to ventricular contractile rates (AV ratio) should equal 1, but will increase if

electrical conduction through the AV node is impaired. The cardiovascular system, and particularly the cardiac zERG (or human equivalent – hERG) channel, is known to be susceptible to impairment by oxidative stress (Zhang et al., 2006; Vandenberg, 2010). It has been demonstrated that deletion of delayed rectifier potassium channels (zERG) in zebrafish can lead to AV block as indicated by increased atrial/ventricular ratio (Langheinrich et al., 2003) as well as cardiac arrhythmias (Sanguinetti and Tristani-Firouzi, 2006). In the current study, because the AV ratio was not significantly different, but ventricular rate was significantly decreased, we cannot exclude the possibility that AV blockade might have been detected with a higher sample size, but future studies are needed (Andersson et al., 2011).

In order to investigate the underlying oxidative stress hypothesis, specific mRNA markers of interest were investigated. Glutathione-s-transferases (GST) are a family of detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Nimmo, 1987), and mRNA transcript abundance of heart GST-pi class was significantly down-regulated, without any change seen in GPX1A expression in zebrafish exposed to 10.3 µg Se/g d.m. Since catabolism of Se-Met has been reported to cause oxidative stress in fish (Palace et al., 2004), we would predict an upregulation of GST-pi if the zebrafish heart was able to compensate for oxidative stress. Since GST-pi instead decreased in cardiac tissue, but not liver, this suggests the heart may have been particularly vulnerable to increased damage from reactive oxygen species.

A second possible explanation for the observed Se-Met-induced cardiac impairment could be due to ventricular fibrosis. The significant increase in echodensity at the

junction between the atrium and ventricle surrounding the AV valve are consistent with changes reported in human diabetic hearts, which was associated with cardiomyopathy and increased fibrosis or stiffening (Dibello et al., 1995). Fibrotic cardiac tissue would impair blood movement from the atrium to the ventricle, resulting in reduced end-diastolic volume (EDV), and this agrees with observations in the current study. The decrease in blood velocity through the AV valve during atrial ejection coupled with the observed decreases in stroke volume and cardiac output in the highest Se-Met group suggests that contractility may also be impaired by the process in zebrafish. Furthermore, significant down-regulation of MMP2 mRNA transcript abundance was observed in hearts of the elevated Se-Met exposed fish. MMPs are a class of proteolytic enzymes that have important vascular and cardiac remodeling properties (Seliktar et al., 2001). Decreased cardiac MMP expression has been reported along with increased collagen content (fibrosis) specifically in rainbow trout (Keen et al., 2016) and this fibrosis is also seen in human diabetic hearts (Russo and Frangogiannis, 2016). Although no previous information is known regarding cardiac remodelling or fibrotic processes, impaired cardiac function, or arrythmias after Se exposure in adult fish, this clearly requires further investigation.

The Se diets in the present study were environmentally relevant, and were based on previous reports of sublethal toxicities in adult zebrafish (Thomas and Janz, 2011; Thomas et al., 2013; Raine et al., 2016). In addition, similar whole body Se concentrations have been reported in fish collected from Se impacted sites (Lemly, 1997; Fan et al., 2002; Hamilton, 2004; Muscatello et al., 2006; Muscatello and Janz, 2009). Whole body Se concentrations increased in a dose-dependent manner, and the 28.8 µg

Se/g d.m. treatment (9.2 µg Se/g d.m.) was slightly greater than the 2016 United States Environmental Protection Agency (USEPA) whole body criterion of 8.5 µg Se/g d.m. (USEPA, 2016). Previous studies reported similar whole-body concentrations in zebrafish exposed to dietary Se-Met (Thomas and Janz, 2011). In addition, liver mRNA transcript abundance of selenoprotein P (SEPP1a) was significantly up-regulated in the 28.8 µg Se/g d.m. treatment compared to the control. SEPP1a is synthesized in the liver and acts as the primary Se transport protein, binding approximately 50% of circulating plasma Se (Papp et al., 2007; Gladyshev, 2012). Thus, it appears that the dose-dependent increase in SEPP1a mRNA transcript abundance amongst treatments is related to elevated Se transport in the bloodstream.

In addition to the cardiovascular effects of elevated dietary Se-Met exposure, we observed elevated levels of stored glycogen in fish fed greater than 10.3 µg Se/g d.m. This could be due to impaired energy homeostasis, caused by modifications of aerobic and metabolic enzyme activities (McGeer et al., 2000; Scott et al., 2002). In order to investigate potential mechanisms of metabolic dysfunction, mRNA transcript abundance of key aerobic energy metabolizing enzymes, citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD) were determined. HOAD is an important mitochondrial enzyme involved in β-oxidation of fatty acids, whereas CS is a key enzyme involved in the citric acid cycle and is used as an index of aerobic metabolic activity (Rajotte and Couture, 2002). In the present study, elevated dietary Se-Met exposure resulted in significant up-regulation of liver CS mRNA transcript abundance in the 28.8 µg Se/g d.m. treatment group. Furthermore, a significant down-regulation of both liver and muscle HOAD mRNA transcript abundance was observed in both elevated dietary

Se-Met treatments. This change in gene expression, along with the increased glycogen stores observed in the highly exposed fish, may be indicative of an impaired ability to use both glycogen and lipid energy stores in these tissues. While CS expression increased in liver, glycogen stores were also increased suggesting that enzymes upstream of CS were impaired, leading to an inability to utilize this energy store. Further studies should examine expression or activity of glycogen phosphorylase and glycogen synthase in Se-Met treated fish to answer this question.

Fish exposed to excess Se in the form of selenite or Se-Met have shown impaired cellular methylation (Ma et al., 2012; Thomas et al., 2013). In addition to protein dysfunction and oxidative stress, methylation could potentially be another mechanism of selenium toxicity. In the present study we observed down-regulation of hepatic MAT1A mRNA transcript abundance following elevated Se-Met exposure. MAT1A belongs to a family of enzymes that regulate the biosynthesis of S-adenosylmethionine (SAM), the principal methyl donor, and reduced MAT1A expression is a marker of both reduced SAM production and impaired methylation (Lu, 2000; Mato et al., 2002). Methylation is known to be important in gene expression but also energy homeostasis (Lu, 2000; Mato et al., 2008), and reduced methylation has been shown to cause increases in hepatic energy stores in fish (Espe et al., 2010). In addition, changes in the epigenome are known to be associated with the onset and progression of cancer, autoimmune diseases, type 2 diabetes and, more notably, cardiovascular diseases (Arai and Kanai, 2010; Timp and Feinberg, 2013; Dozmorov et al., 2014; Nilsson et al., 2014; Whayne, 2014). Although our results were consistent with those found in previous Se exposure studies in fish and mammalian liver (Hasegawa et al., 1996; Zeng et al., 2011; Thomas et al., 2013), other

studies have found opposing results of increased methylation with increased Se exposure (Davis et al., 2000; Uthus et al., 2006). Further controlled experimental studies are needed to characterize whether excess Se exposure alters down-stream methylation reactions.

The authors acknowledge that one of the major limitations in the present study was the lack of replicated tanks for each treatment. However, the goal was not to examine population level effects that would require replicated tanks for statistics. While replication is needed to ensure that treatment effects and not random tank effects are reported, the present study saw a clear dose-response relationship within several different end-points, allowing for the consideration that these results are true treatment effects, and not random tank effects.

2.6 Conclusion

In conclusion, the present study demonstrated that exposure to environmentally relevant concentrations of dietary Se-Met can cause significant cardiotoxicity and impaired energy homeostasis in adult zebrafish. Se-Met exposure caused significant increases in echodensity consistent with fibrosis surrounding the AV valve, with accompanying reductions in stroke volume and cardiac output. In addition to the direct physiological effects, Se-Met produced effects at the cellular level, causing down-regulation of key methylation (MAT1A), vascular remodeling (MMP2) and metabolic (HOAD) gene expression. Based on the results of this study, a proposed adverse outcome pathway is summarized in Figure 2.7. Overall, these effects would impair the aerobic capacity of fish and have potential ecological consequences on overall fitness.

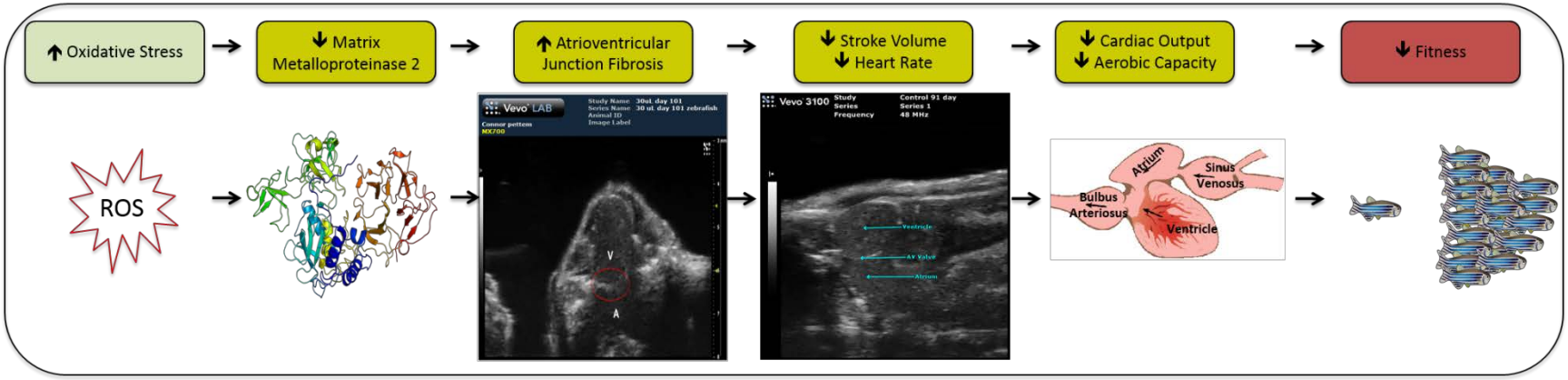

Figure 2.7. Hypothesized adverse outcome pathway (AOP) linking dietary selenomethionine exposure with adverse cardiorespiratory toxicity.

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CHAPTER 3

3.0 CARDIAC AND METABOLIC EFFECTS OF DIETARY SELENOMETHIONE EXPOSURE IN RAINBOW TROUT

Preface

The research in this chapter was designed to address the underlying cardiovascular and metabolic implications of dietary selenomethionine exposure in juvenile rainbow trout.

Chapter 3 of this thesis has been submitted to Aquatic Toxicology for publication (November 2017) and is currently in review.

Pettem, C.M., Briens, J.M., Janz, D.J., and Weber, L.P., (2017). Juvenile Rainbow Trout (*Oncorhynchus mykiss*) Become Glucose Intolerant and Develop Diabetes-Related Complications Following Dietary Selenomethionine Exposure. *Aq. Toxicol* (AQTOX-D-17-00814).

The author contributions to chapter 2 of this thesis were as follows:

Connor Pettem (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

Lynn Weber (University of Saskatchewan) helped design the study, provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

Jennifer Briens (University of Saskatchewan) helped by performing the High Performance Liquid Chromatography (HPLC work to analyze plasma samples for methylglyoxal concentrations.

3.1 Abstract

Selenium (Se) is considered as an essential trace element, involved in important physiological and metabolic functions for all vertebrate species. Fish require dietary concentrations of 0.1 to 0.5 μg Se/g dry mass (d.m.) to maintain normal physiological and selenoprotein function, however concentrations exceeding 3 μg/g d.m. have been shown to cause toxicity. As Se is reported to have a narrow margin between essentiality and toxicity, there is growing concern surrounding the adverse effects of elevated Se exposure caused by anthropogenic activities. Previous studies have reported that elevated dietary exposure of fish to selenomethionine (Se-Met) can cause significant cardiotoxicity and alter aerobic metabolic capacity, energy homeostasis and swimming performance. The goal of this study aims to further investigate mechanisms of sublethal Se-Met toxicity, particularly potential underlying cardiovascular and metabolic implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met in juvenile rainbow trout (*Oncorhynchus mykiss*). Juvenile rainbow trout were fed either control food (1.3 μg Se/g dry mass [d.m.]) or Se-Met spiked food (6.4, 15.8 or 47.8 μg Se/g d.m.) for 60 d at 3% body weight per day. Following exposure, ultrahigh resolution B-mode and Doppler ultrasound was used to characterize cardiac function *in vivo*. Chronic dietary exposure to Se-Met significantly increased stroke volume, cardiac output, and ejection fraction. Fish fed with Se-Met spiked food had elevated liver glycogen and triglyceride stores, suggesting impaired energy homeostasis. Exposure to Se-Met significantly decreased mRNA abundance of citrate synthase (CS) in liver and serpin peptidase inhibitor, clad H1 (SERPINH) in heart, and increased mRNA abundance of sarcoplasmic reticulum calcium ATPase (SERCA) and key cardiac remodelling

enzyme matrix metalloproteinase 9 (MMP9) in heart. Taken together, these responses are consistent with a compensatory cardiac response to increased susceptibility to oxidative stress, namely a decrease in ventricular stiffness and improved cardiac function. These cardiac alterations in trout hearts were linked to metabolic disruption in other major metabolic tissues (liver and skeletal muscle), impaired glucose tolerance with increased levels of the toxic glucose metabolite, methylglyoxal, development of cataracts and prolonged feeding behaviour, indicative of visual impairment. Therefore, although juvenile rainbow trout hearts were apparently able to functionally compensate for adverse metabolic and anti-oxidant changes after chronic dietary exposure Se-Met, complications associated with glucose intolerance in mammalian species were evident and would threaten survival of juvenile and adult fish.

3.2 Introduction

Selenium (Se) is considered as an essential trace element, involved in important physiological and metabolic functions for all vertebrate species. Fish require dietary concentrations of 0.1 to 0.5 μg Se/g dry mass (d.m.) to maintain normal physiological and selenoprotein function, however concentrations exceeding 3 μg/g d.m. have been shown to cause toxicity (Janz, 2012). This represents the paradoxical nature of Se, as there is a narrow margin between essentiality and toxicity. Oviparous vertebrates such as birds and fishes are among the most sensitive organisms, as selenium is deposited into eggs during vitellogenesis, and transferred to developing embryos during yolk resorption (Lemly, 1997; Janz et al., 2010). Selenium is a naturally occurring element, commonly found in areas of sedimentary rock depositions, in particular black shale, phosphate and coal deposits (Presser et al., 2004; Maher et al., 2010). It naturally enters aquatic environments

in relatively low concentrations, providing fish and other aquatic organisms with the adequate levels needed to maintain normal metabolic functions (Maher et al., 2010). However, coal-fired power plants, petroleum refineries, as well as agricultural and mining industries, can greatly exacerbate the loading of Se into the environment by manufacturing waste generation (Lemly, 2004; Maher et al., 2010; Janz, 2012). The adverse effects of excessive Se in fish are well documented in laboratory experiments and are evident in numerous field based studies (Janz et al., 2010). Water soluble inorganic Se, in the form of selenite and selenate, are taken up by primary producers and microorganisms, then biotransformed into organoselenoproteins, predominantly selenomethionine (Se-Met) and selenocysteine (Se-Cys) (Fan et al., 2002). Upon ingestion, there is a trophic transfer of organic Se amongst primary consumers, secondary consumers and finally higher order consumers including birds, fish, and humans. As a result, there is a risk of bioaccumulation and biomagnification of organic Se. Se-Met is the predominant dietary source of Se available to fish (Fan et al., 2002). Due to the structural similarity between Se-Met and the amino acid methionine, Se-Met can avoid biotransformation and be directly integrated into any methionine-containing protein in a non-specific, dose-dependent manner (Behne et al., 1991). In addition, due to its biotransformation to selenoxides, such as methylselenol, excessive Se-Met uptake has been shown to cause oxidative stress *in vitro* (Palace et al., 2004; Spallholz et al., 2004). While the underlying toxicodynamic mechanisms behind Se toxicity are not fully understood, it is generally agreed that protein dysfunction and oxidative stress may be the fundamental culprits (Ma et al., 2012).

Using zebrafish (*Danio rerio*) as a model test organism, elevated exposure to Se-Met has been reported to cause significant cardiotoxicity as well as alter aerobic metabolic capacity, respiration, energy homeostasis, swimming performance and increased incidence of early life stage deformities and mortalities (Tashjian et al., 2006; Thomas and Janz, 2011; Thomas et al., 2013; Arnold et al., 2016; Pettem et al., 2017). Conversely, there is a lack of research investigating the potential role of cardiovascular dysfunction associated with such responses in rainbow trout exposed to elevated chronic dietary Se exposure. Previous studies have shown that the oxygen consumption (MO_2) of fish exposed to Se were consistently greater than that of control fish (Scott and Sloman, 2004; Thomas and Janz, 2011; Thomas et al., 2013). Metabolic rate and cardiac output are closely integrated, allowing the heart to adjust for increases in oxygen demand, and any alterations to these responses could lead to impaired aerobic performance (MacKinnon and Farrell, 1992). Whether the Se-Met-mediated increases in oxygen demand can be met by increased cardiac output in juvenile trout is unknown. Advances in high frequency ultrasound bio-microscopy have proven to be an accurate, non-invasive approach to visualize cardiac function *in vivo* in adult fish, allowing for real-time detailed imaging of cardiac structures and blood flow characterizations (Gerger et al., 2015; Gerger and Weber, 2015; Pettem et al., 2017). Natural and anthropogenic stressors have been shown to alter both metabolic energy expenditure and storage, as well as impair the physiological stress response in fishes (Mommsen et al., 1999; McKenzie et al., 2007; Thomas and Janz, 2011; Wiseman et al., 2011; Thomas et al., 2013). Thus, impairment of these key physiological responses may cause a downstream cascade effect on important physiological functions, including the cardiovascular system. In a previous study using

zebrafish and similar diets, a significant down-regulation of matrix metalloproteinase 2 (MMP2) mRNA transcript abundance was observed in hearts of the elevated Se-Met exposed fish (Pettem et al., 2017). MMPs are a class of proteolytic enzymes that have important vascular and cardiac remodelling properties (Seliktar et al., 2001), and decreased cardiac MMP expression has been reported along with increased collagen content (fibrosis) specifically in rainbow trout (Keen et al., 2016) and this fibrosis is also seen in human diabetic hearts (Russo and Frangogiannis, 2016).

Glycogen and triglycerides are the two main forms of energy stored in fish and are predominantly used in burst and prolonged swimming, respectively (Hammer, 1995; Moyes and West, 1995). The mobilization of these energy stores are important in not only swimming, growth, and reproduction, but also have a pivotal role in the detoxification of xenobiotics (Bennett and Janz, 2007). Fish fed a range of excess Se-Met in controlled laboratory settings, as well as those collected from Se-contaminated sites, exhibited elevated levels of stored triglycerides and glycogen (Bennett and Janz, 2007; Wiseman et al., 2011; Thomas et al., 2013; Pettem et al., 2017). In animal models of human disease, excess dietary selenium has been shown to impair insulin-regulated carbohydrate and lipid metabolism through a mechanism involving reactive oxygen species to produce diabetes (Steinbrenner, 2013; Wang et al., 2014). Moreover, prolonged hyperglycemia is associated with increased circulating levels of the toxic glucose metabolite, methylglyoxal, in mammals (Desai et al., 2010; Adolphe et al., 2012; Kalapos, 2013). Methylglyoxal, in turn, is known to promote oxidative stress and is a precursor to advanced glycation end products responsible for development of diabetic complications such as cataracts (Desai et al., 2010; Kalapos, 2013). In fish, even in the

absence of overt diabetes, inadequate mobilization of these energy stores could lead to impaired swimming and cardiovascular impairment. Whether a similar link exists between excess selenium, impaired energy mobilization, cardiotoxicity and development of impaired glucose control in fish is unclear.

The overall goal of this study was to further investigate sublethal mechanisms of Se toxicity in juvenile rainbow trout, particularly potential underlying cardiometabolic and energy storage implications of chronic exposure to environmentally relevant concentrations of dietary Se-Met. Cardiac function was assessed *in vivo* using ultrahigh resolution ultrasonography, followed by quantification of energy stores in heart, liver and muscle tissues, and mRNA transcript abundance of selected genes involved in aerobic metabolism, cardiac function, and oxidative stress. Glycemic control was assessed using an intraperitoneal glucose challenge, with blood glucose and levels of the toxic glucose metabolite, methylglyoxal, measured. Finally, fish were evaluated for development of cataracts and behavioural evidence of visual impairment.

3.3 Materials and Methods

3.3.1 Test compound

Seleno-L-methionine (≥ 98% purity) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2 Test species

All female rainbow trout eggs were purchased from a commercial supplier (Troutlodge, Sumner, WA, USA) and reared in the Aquatic Toxicology Research Facility at the Toxicology Centre, University of Saskatchewan. Eggs were hatched and fish reared

for 12 months, then 240 juvenile (yearling) rainbow trout (size ranging from 80-100 g) were randomly selected from this colony and placed into four 719L tanks (60 fish/tank). These tanks were supplied with continuous aeration (11 ± 0.5 mg/L dissolved oxygen), running water with a flow rate of 3.5L/min, controlled temperature (12 ± 1ºC) and photoperiod (14 h light: 10 h dark). Fish were then acclimated for 2 weeks to these conditions and fed commercial trout pellet food (Martin Classic Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada) twice daily prior to the beginning of the dietary exposure with daily 50% water changes. Experiments were conducted according to procedures approved by the University of Saskatchewan Animal Care and Use committee according to the Canadian Council on Animal Care guidelines.

3.3.3 Diet preparation

Nominal concentrations (3, 10 and 30 µg/g d.m.) of Se in the form of Se-Met were dissolved in nanopure deionized distilled water, added to ground trout pellets, and mixed thoroughly for approximately 20 min. The control diet followed the above process with an equal volume of water, without the addition of Se-Met. Diets were then fed through a meat grinder, cut into individual pellets and placed in a 55°C drying oven for 24 h until water had been removed from all the diets. Food was then stored at 4°C for the remainder of the experiment. Representative samples of each diet were taken for total Se analysis using inductively coupled plasma-mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada).

3.3.4 Feeding exposure, behavioural test, morphometric analyses and cataract evaluation

Each treatment group consisting of 60 fish was fed twice daily (3% body weight/day) with either control or Se-Met spiked diets for 60 d. Fish were given approximately 1 h to feed, after which all excess waste was siphoned, and 50% water renewals were performed daily. Following 7, 14, 28 and 56 d of dietary exposure, n = 3 fish from each treatment were randomly selected, euthanized with an overdose of tricaine methanesulfonate (MS 222) (1g/L) followed by spinal dislocation, muscle tissues harvested, and stored at -80°C for subsequent muscle Se bioaccumulation analysis. In addition, n = 15 fish from each treatment were randomly selected for cardiac ultrasonography as described in section 2.6. Fish from each treatment group (n = 10) were randomly selected and euthanized, and liver, heart and muscle samples were harvested for subsequent analysis of triglyceride and glycogen storage. Additionally, n = 5 fish from each treatment had heart, liver and muscle tissues harvested and placed in -80°C storage and were used for mRNA analysis of genes of interest using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Prior to dissection and storage, fish morphometrics (total length, body weight, condition factor, hepatosomatic index (HSI), cardiosomatic index (CSI) and incidence of cataracts) were determined for all fish. Additionally, the prevalence of cataracts were counted in each treatment group prior to euthanization and expressed as a percent incidence. Furthermore, as a surrogate for visual testing, feeding behaviour was assessed using rainbow trout ability to actively forage for food following 60 d of dietary exposure as outlined in section 3.2.9. Finally, n = 12 fish from each treatment were used to determine glycemic control using a glucose tolerance test, as outlined in section 3.2.9.

3.3.5 Total Se analysis

Food and rainbow trout muscle samples were lyophilized using a freeze dryer and homogenized using a tissue homogenizer (BioSpec Products, Bartlesville, OK). 100 mg aliquots were cold digested in Teflon vials with 5 ml of ultra-pure nitric acid and 1.5 ml of hydrogen peroxide. Upon digestion, samples were evaporated on a hot plate (<75°C), reconstituted in 5 ml of 2% ultrapure nitric acid, and stored at 4°C until ICP-MS analysis could be performed to determine total Se concentrations. In addition to tissue samples, water aliquots from each aquaria were taken to determine aqueous Se concentrations, and were found to not differ amongst treatments. Therefore, any differences among treatments observed can be attributed solely to dietary, not aqueous, exposure to Se. A detection limit of 0.6 µg Se/g d.m. was determined using method blanks, and the recovery of Se (92.4 ± 0.5%) was determined using a certified reference material (TORT-3, lobster hepatopancreas, NRC, Ottawa, ON, Canada). All total Se concentrations in diets and tissues are presented on a dry mass basis.

3.3.6 Cardiovascular endpoint evaluation

In vivo cardiac imaging was performed using non-invasive ultrahigh frequency ultrasonography in order to quantify cardiovascular function. This technique was adapted from a rodent model to rainbow trout, using a VEVO 3100 high frequency ultrasound (VisualSonics, Markham, ON, Canada) capable of B-mode imaging and pulse-wave Doppler (VisualSonics, 2015) to quantify cardiac function according to methods previously published by our laboratory in zebrafish (Gerger et al., 2015; Gerger and Weber, 2015; Pettem et al., 2017). Prior to ultrasonography, fish anesthesia was initiated in an aerated bucket of dechlorinated water containing 25 mg/L of Aquacalm

(metomidate hydrochloride). After loss of righting reflex, fish were placed ventral side up in a foam holder submerged in a container with continuous flow of highly aerated water with light anesthesia (10 mg/L) of Aquacalm. Water temperature for rainbow trout during anesthesia was maintained at 12 ± 0.7 ˚C (same as housing temperature) using a recirculating, refrigerated water bath (VWR International, Mississauga, ON, Canada). A handheld transducer was then used to produce acoustic pulses up to 75 MHz toward the body cavity of rainbow trout, producing real-time *in vivo* short-axis and long-axis imaging at a resolution of 30 μm. B-mode images were collected and used to determine outer ventricular volume at systole and diastole (minimum of 3 images for each view at each stage of the cardiac cycle averaged per fish) using Simpson's rule of discs (Mercier et al., 1982; VisualSonics, 2015). Briefly, three different short axis views of the ventricle were used to calculate circular area (A1, A2, A3). The ventricular length (l) from a long axis view was then measured and divided by the total number of short axis views (3) to give the height (h=1/3) of each ventricular disk and used to calculate end systolic (ESV) and diastolic volumes (EDV) using the following equation:

$$V = (A1+A2)h + ((A3h)/2) + (\pi/6(h3)) \qquad (1)$$ (Mercier et al., 1982)

End systolic volume was then subtracted from end diastolic volume to give ventricular stroke volume (VS). All volumes (EDV, ESV, VS) and cardiac output data were also corrected for individual fish body weight.

$$VS = EDV - ESV \qquad (2)$$

Ventricular and atrial contractile rates were calculated by counting individual beats in 5 second B-mode ultrasound video loops, then converting to beats per minute (BPM). Cardiac output (Q) was then calculated using ventricular contractile rate (fH) and (VS):

$$Q = \text{Ventricular } f\text{H} \times V\text{S} \qquad (3)$$

Finally, in order to evaluate efficiency of electrical excitation travelling from the atrium to the ventricle through the atrioventricular (AV) node, atrial contractile rate was divided by ventricular contractile rate to calculate the AV ratio. In ideal physiological conditions, this value should be equal to 1.0:

$$\text{AV Ratio} = \text{Atrial } f\text{H} \ / \ \text{Ventricular } f\text{H} \qquad (4)$$

3.3.7 Determination of triglyceride and glycogen stores

Concentrations of heart, muscle and liver triglycerides were determined upon completion of the 60 d exposure using a commercially available kit (Sigma-Aldrich, Oakville, ON, Canada) adapted and previously validated use in whole fish homogenates in our laboratory (Weber et al., 2003; Bennett and Janz, 2007).

Concentrations of heart, muscle and liver glycogen were analyzed upon completion of the 60 d exposure using methods previously validated in our laboratory (Weber et al., 2008). Standard curves were prepared using purified Type IX bovine liver glycogen. Standard and reagents for the assay were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.8 Gene expression analysis

Abundance of mRNA transcripts of genes coding for proteins of interest were quantified using qRT-PCR. Total RNA was extracted from muscle using RNeasy Lipid Tissue Mini Kit (Qiagen, Mississauga, ON, Canada) and from liver or heart using the RNeasy Plus Mini Kit (Qiagen). Purified total RNA was then quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE,

USA). The purified RNA samples were stored at −80 ºC until synthesis of cDNA. First-strand cDNA synthesis was performed using a QuantiTect® Reverse Transcription Kit (Qiagen) from 1 μg total RNA. The cDNA samples were stored at −20ºC until further analysis. qRT-PCR was performed in 96-well plates by use of a QuantStudio 6 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sequences of gene specific primers were designed against target genes by use of PrimerBlast software (NCBI Biosystems, Bethesda, MD, USA). A separate 45 μL PCR reaction mixture consisting of Power SYBR Green master mix (Applied Biosystems), cDNA, gene specific primers, and nuclease free water was prepared for each cDNA sample and primer pair. A final reaction volume of 20 μL was transferred to each well and reactions were performed in duplicate. The PCR reaction mixture was denatured at 95ºC for 10 min before the first PCR cycle. The thermal cycle profile was performed as follows: denature for 10 s at 95ºC and extension for 1 min at 60ºC for a total of 40 PCR cycles. Optimal PCR conditions were established by determining the efficiency of each PCR assay with a standard curve of serially diluted cDNA standards. Target gene mRNA abundance was quantified by normalizing to the expression of elongation factor 1α (EF 1α) according to the Mean Normalized Expression (MNE) method of Simon (2003).

3.3.9 Intraperitoneal glucose tolerance test, plasma methylglyoxal determination, cataract scoring and behavioural testing

Rainbow trout were fasted for 24 h, then n = 6 from each treatment were segregated to individual pens within a flow-through 379 L tank. Fasting blood measurements at time 0 (baseline) were taken by collecting ~5 μL of blood (one drop) from the caudal peduncle using a syringe and whole blood transferred directly onto a glucose indicator strip

connected to a OneTouch Ultra 2 glucometer (LifeScan Canada Ltd., Burnaby, BC, Canada) to measure blood glucose. For all blood glucose measurements at each time-point, averaged values from minimum three strips with good agreement were used to ensure precision. Following baseline, fish were injected intraperitoneally (i.p.) with a glucose solution (0.5g/kg; 10% glucose in deionized water) using a 25 G needle. Fish were then returned to their holding pen. Blood samples were then taken at 0.5, 1, and 3 h from 6 fish, then 6, 12, 24 and 48 h, from another 6 fish, following the above protocol. Preliminary experiments in untreated rainbow trout showed that a single fish could not be used to sample all of the time-points needed without excessive stress (excessive pallor and loss of activity). When only half the time-points per fish were used instead, fish behaviour and appearance remained normal throughout the sampling period. At 48 h after i.p. glucose injection, fish were euthanized with an overdose of tricaine methanesulfonate (MS 222) (1g/L).

Plasma methylglyoxal was measured using HPLC according to methods adapted from that used previously in dog plasma in our laboratory (Adolphe et al., 2012). Frequency of cataracts were scored for each treatment prior to euthanization and reported as a percent prevalence in each tank. As a surrogate for vision testing, feeding behaviour was assessed using rainbow trout ability to actively forage for food following 60 d of dietary exposure. One scoop (67.5 g) of either control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (6.4, 15.8 and 47.8 μg Se/g d.m.) was distributed throughout the tank within a 5 second period, then the timer began. The time taken for all the fish in a tank to return to normal swimming behaviour after feeding was recorded. Since only a single tank per treatment

was used in this study, both cataract incidence and feeding behaviour end-points are reported as a qualitative results with no statistical analyses.

3.3.10 Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test and homogeneity of variance with the Levene test using SYSTAT 11 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) post hoc test was used to determine significant differences among Se-Met treatments from the control group. All statistical tests used an alpha value of 0.05 to be considered statistically significant. Data are presented as mean ± standard error of the mean (SEM).

3.4 Results

3.4.1 Selenium analysis

Total Se concentrations in food are shown in Table 3.1. Total Se concentration in the control diet was 1.3 ± 0.01 µg Se/g d.m. and in the Se-Met spiked food (nominal concentrations 3, 10 and 30 µg Se/g d.m.) were 6.4 ± 0.35, 15.8 ± 0.37 and 47.8 ± 2.5 µg Se/g d.m. respectively, which were all significantly greater than the control diet ($p < 0.05$, Table 3.1). Muscle Se concentrations in rainbow trout fed 1.3, 6.4, 15.8 and 47.8 µg Se/g d.m. were measured following 7, 14, 28 and 56 d of dietary exposure. Dose and time-dependent increases in Se were clearly evident, with significantly greater concentrations seen in fish fed 15.8 and 47.8 µg Se/g d.m. starting at 14 d of feeding, compared to control ($p < 0.05$, Figure 3.1).

Table 3.1. Mortality, total length, body weight, condition factor, hepatosomatic (HSI) and cardiosomatic (CSI) indices in juvenile rainbow trout fed different concentrations of selenomethionine for 60 d. Cumulative mortalities were calculated throughout 60 d exposure and are a percentage of the initial tank population (n = 1 tank/treatment; no statistics performed on mortality), while morphometrics were determined on day 60 (n = 40-50 fish/treatment). Dietary selenium was determined in 3 random samples from each diet (n = 3). Data are mean ± S.E.M.

Dietary [Se] (µg/g dry mass)	Mortality (%)	Total Length (cm)	Body Weight (g)	Condition Factor	HSI	CSI
1.3 ± 0.00	6.5	26.5 ± 0.3	239.5 ± 8.6	1.26 ± 0.02	1.06 ± 0.01	0.143 ± 0.01
6.4 ± 0.35	7.4	24.1 ± 0.4	184.4 ± 7.7*	1.28 ± 0.01	0.95 ± 0.02	0.155 ± 0.01
15.8 ± 0.37*	12.1	25.4 ± 0.4	216.2 ± 8.9	1.29 ± 0.01	1.00 ± 0.03	0.146 ± 0.01
47.8 ± 2.50*	8.4	25.5 ± 0.4	220.0 ± 11.7	1.28 ± 0.02	1.39 ± 0.06*	0.142 ± 0.01

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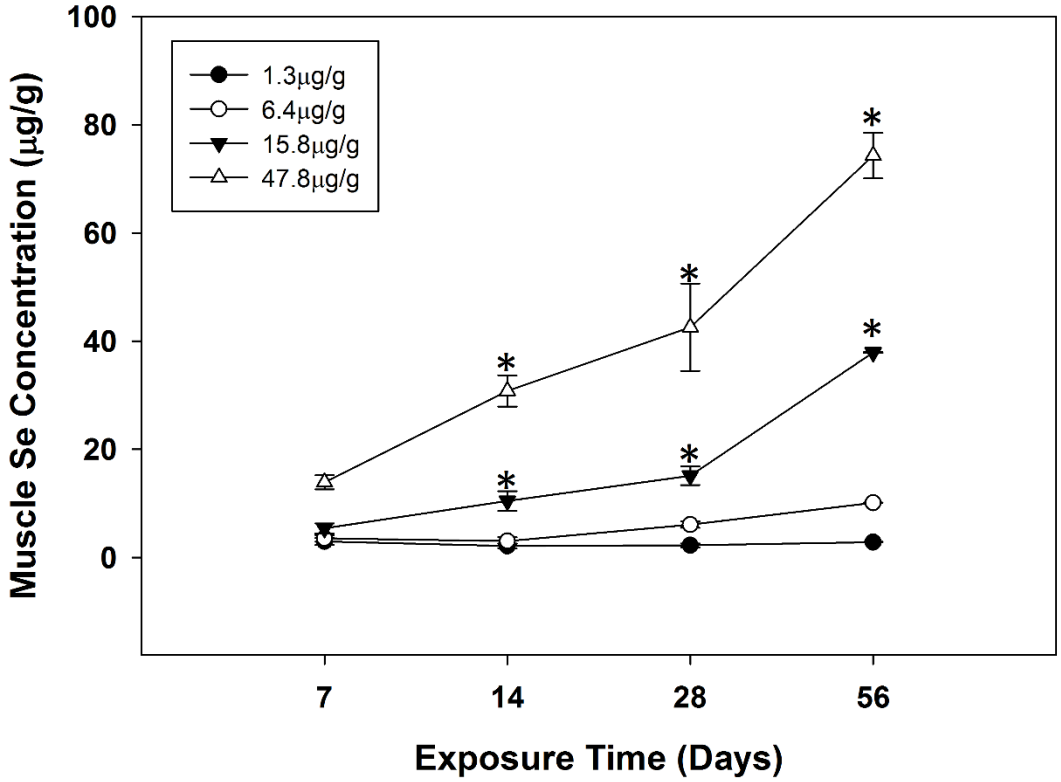

Figure 3.1. Muscle selenium concentrations (µg/g d.m.) measured over time in response to increasing inclusion of dietary selenomethionine (Se-Met). * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

3.4.2 Fish mortalities and morphometrics

Mortalities in control, 6.4, 15.8 and 47.8 µg Se/g d.m. fed fish groups after 60 d were similar, being 6.5%, 7.4%, 12.1% and 8.4%, respectively (Table 3.1). Average total fish length in control and Se-Met spiked diets ranged from 24.1 to 26.5 cm and average body mass of fish ranged from 184.4 to 239.5 g, with significantly lesser weights in the 6.4 µg Se/g d.m. fish compared to the control (184.4 ± 7.7, 239.5 ± 8.6, respectively, $p < 0.05$, Table 3.1). Condition factor for control and Se-Met exposed fish ranged from 1.26 to

1.29 (Table 3.1), and did not differ significantly among groups. Fish from the 47.8µg Se/g d.m. treatment had a significantly greater hepatosomatic index (HSI) when compared to the control (1.39 ± 0.06 and 1.06 ± 0.01, respectively, $p < 0.05$, Table 3.1). There were no statistically significant differences in any of the other morphometric measures among exposed and control fish.

3.4.3 Cardiovascular function

Dietary Se-Met exposure had a pronounced effect on juvenile rainbow trout cardiac function. Representative B-mode long axis view and colour Doppler flow sonograms as shown in Figure 3.2 were used to determine all cardiovascular endpoints. Although no significance was observed in end systolic or end diastolic volume among treatments (Figure 3.3 A, B), stroke volume was significantly increased in fish fed 47.8 µg Se/g d.m. (1.09 ± 0.03 µl/g) when compared to control (0.81 ± 0.05 µl/g) (Figure 3.3 C). Ejection fraction was also significantly increased ($p < 0.05$) in fish fed 47.8 µg Se/g d.m. (42.3 ± 1.7%) compared to control (35.4 ± 1.9%) (Figure 3.3 D). Atrial contractile rate was significantly reduced in fish fed 47.8 µg Se/g d.m. (53.6 ± 1.6 BPM) compared to control (62.1 ± 2.4 BPM) (Figure 3.3 E), while ventricular contractile rate (Figure 3 F) and ratio of atrial to ventricular contractile rates (AV ratio; Figure 3.3 G) both remained unchanged. Fish from the highest Se-Met exposure group also showed a significant increase ($p < 0.05$) in cardiac output (60.2 ± 4.7 µl/min/g) when compared to the control group (44.1 ± 3.5 µl/min/g) (Figure 3.3 H). The velocity of blood flowing through the AV valve during active atrial ejection was quantitated using pulsed wave Doppler and a representative Doppler sonogram is shown (Figure 3.4 A). Quantitative analyses of the pulsed wave Doppler sonograms showed that peak velocity of the blood travelling

through the AV valve during active atrial ejection was not statistically different amongst treatments (Figure 3.4 B). However flow through the AV valve was significantly greater ($p < 0.05$) during passive ventricular filling (passive flow from atria to ventricle during atrial diastole) in both the 15.8 and 47.8 µg Se/g d.m. treatment groups (44.1 ± 3.2 and 49.2 ± 1.6 mm/s, respectively) compared to control (35.8 ± 2.3 mm/s) (Figure 3.4 C). Accordingly, a significantly greater ($p <0.05$) passive/active AV velocity was seen in the 47.8 µg Se/g d.m. treatment group (12.8 ± 0.6%) compared to the control rainbow trout (9.0 ± 0.6%) (Figure 3.4 D, $p < 0.05$).

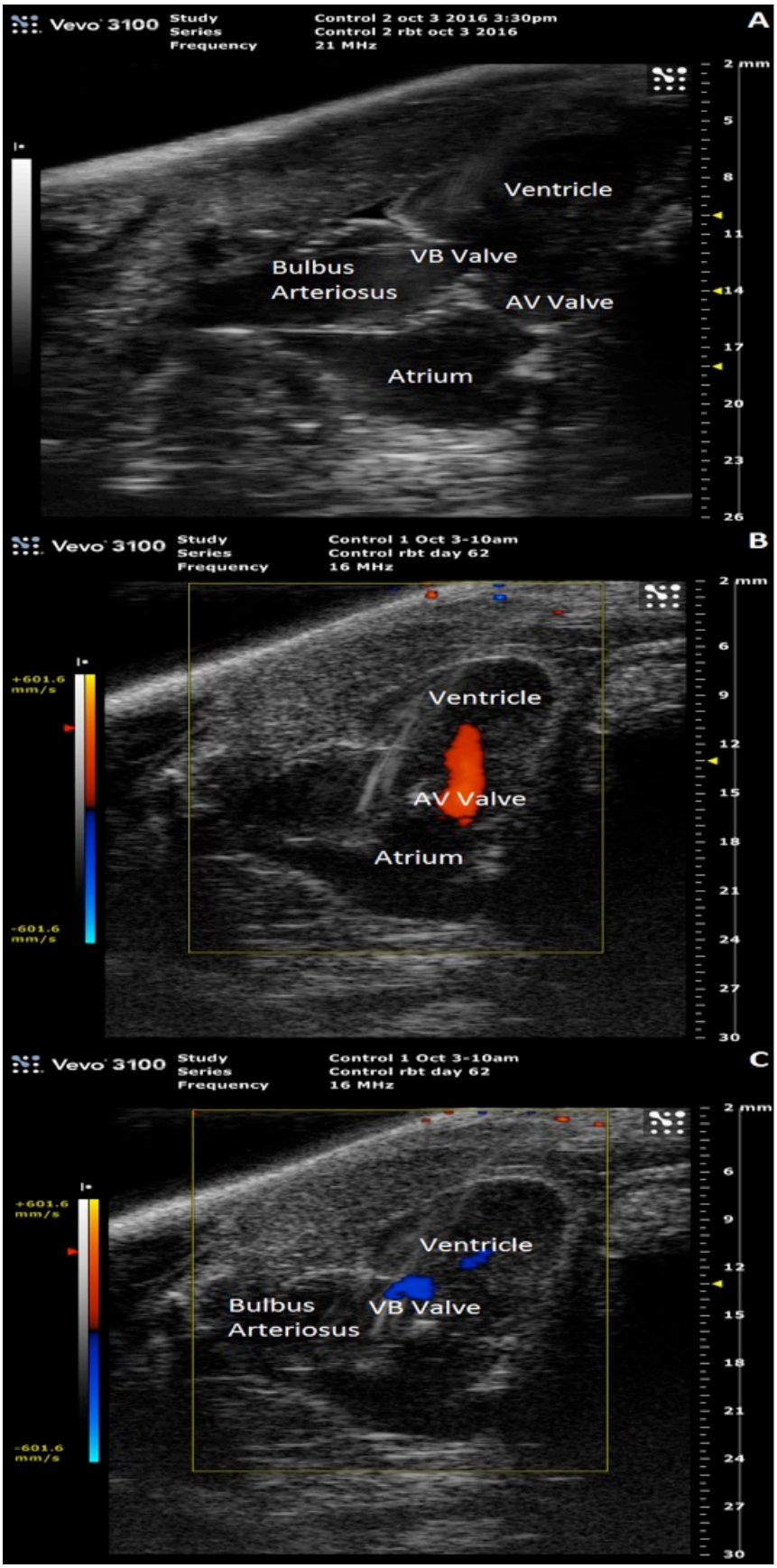
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Figure 3.2. Representative long-axis brightness mode (B-mode) and colour flow Doppler short-axis views of the juvenile rainbow trout heart. Anesthetized control trout are imaged ventral side up, with B-mode showing general cardiac structures indicated by labelled arrows in panel **A** (AV = atrioventricular valve; VB = ventriculobulbar valve). Blood flow direction and velocity are indicated by colour in panel **B**, with red indicating a slow to moderate speed with unidirectional flow from atrium to ventricle through the atrioventricular valve using colour flow Doppler mode. Panel **C** shows blood flow through the ventriculobulbar valve.

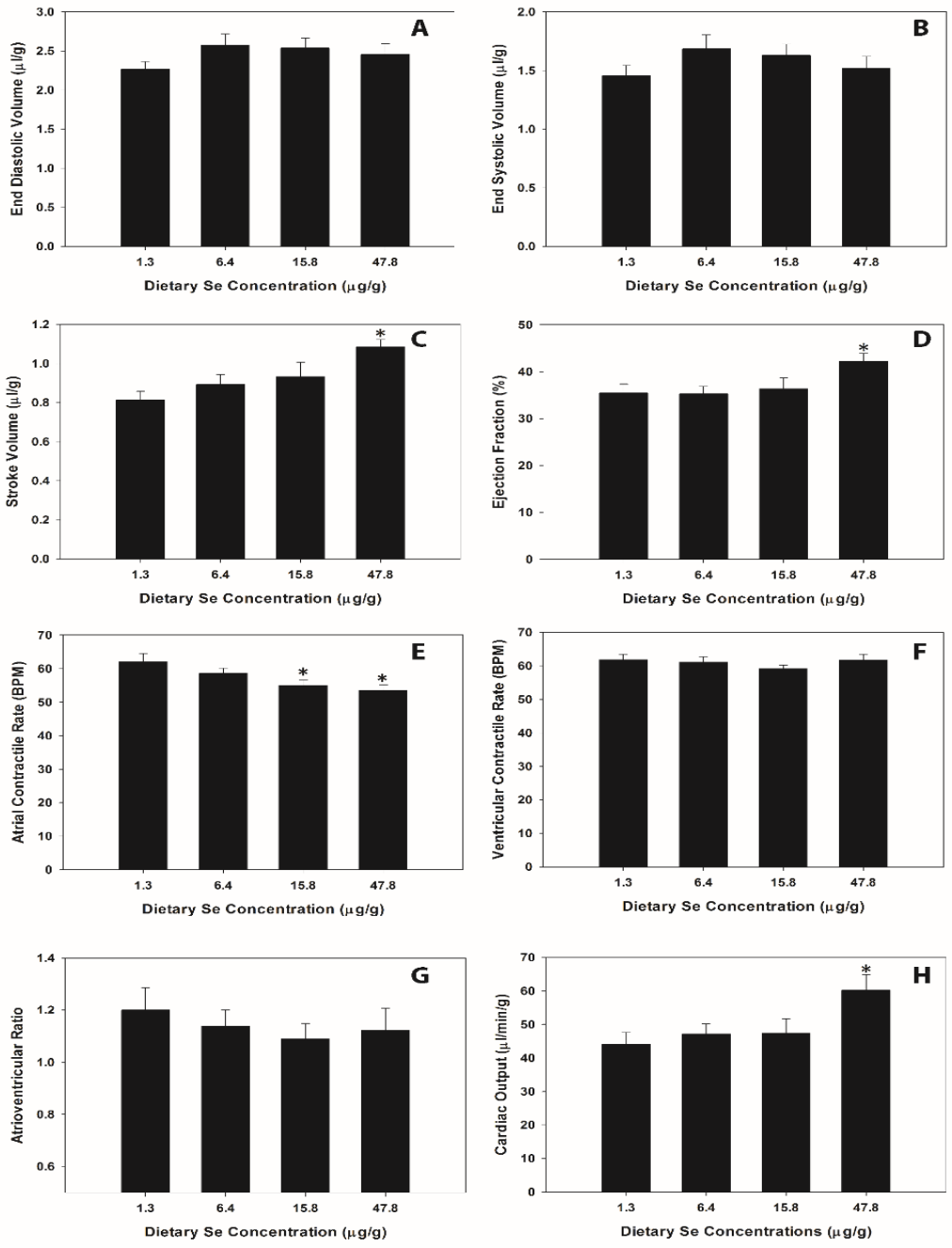

Figure 3.3. Quantitative analyses using cardiac ultrasound in rainbow trout fed increasing levels of selenomethionine. **A**, End diastolic volume, **B**, end systolic volume,

C, stroke volume, **D**, ejection fraction, **E**, atrial contractile rate (beats per minute; BPM), **F**, ventricular contractile rate (BPM), **G**, ratio of atrial to ventricular contractile rates (AV ratio), and **H**, cardiac output of juvenile rainbow trout fed control (1.3 μg Se/g d.m.) or selenomethionine spiked diets (6.4, 15.8 and 47.8 μg Se/g d.m.) for 60 d. Data are expressed as mean ± S.E.M of n = 15 fish/group. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

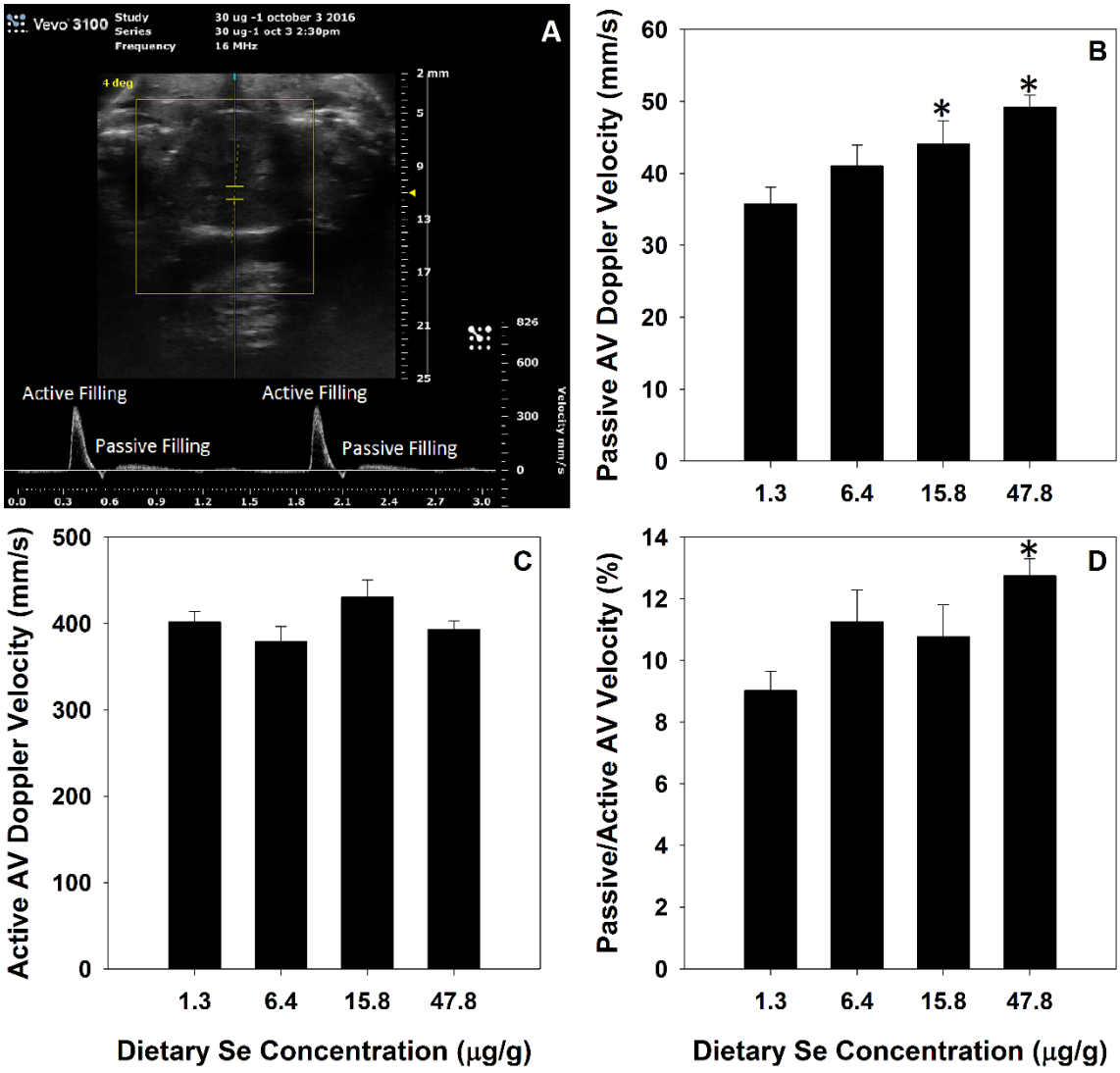

Figure 3.4. Doppler velocity analyses using cardiac ultrasound in rainbow trout. **A,** Representative pulsed wave Doppler at atrioventricular (AV) valve, **B**, passive atrial ejection velocity measured as velocity of passive blood flow movement through the AV valve, **C,** active atrial ejection velocity, and **D**, passive divided by active velocity as a percentage. Data are mean ± S.E.M of n = 15 fish/group. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

A significant increase in heart mRNA transcript abundance of the human Ether-à-go-go-Related Gene (hERG) was observed in the 6.4 µg Se/g d.m. treatment group, but not the higher Se groups, compared to the control ($p < 0.05$, Figure 3.5 A). This gene is crucial in the repolarization of the cell membrane. Additionally, a significant increase in the mRNA transcript abundance of the crucial gene involved in relaxation of cardiac muscle, heart sarcoplasmic reticular calcium ATPase (SERCA), was observed in the highest exposed (47.8 µg Se/g d.m.) group, when compared to the control ($p < 0.05$; Figure 3.5 B). A significant decrease in heart molecular chaperone serpin peptidase inhibitor, clad H1 (SERPINH1) mRNA abundance was also observed in the highest Se dietary group when compared to control fish ($p < 0.05$; Figure 3.5 C). Moreover, there was a significant increase in heart mRNA abundance of the cardiovascular remodelling protein (matrix metalloproteinase 9 [MMP9]) in the 47.8 µg Se/g d.m. dietary group when compared to the control ($p < 0.05$; Figure 3.5 D).

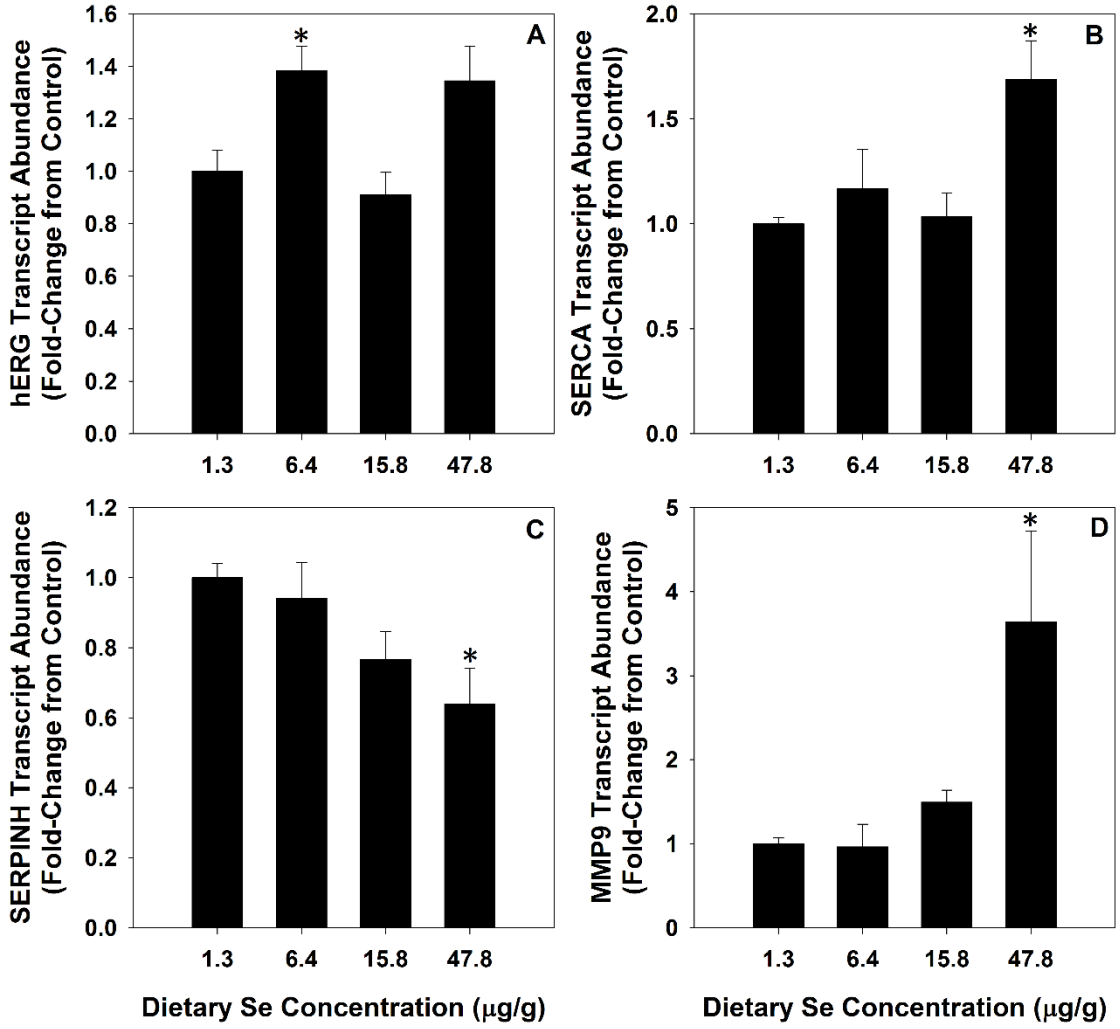

Figure 3.5. Cardiac mRNA abundance of **A**, human Ether-à-go-go-Related Gene (hERG), **B**, sarcoplasmic reticular calcium ATPase (SERCA), **C**, serpin peptidase inhibitor, clad H (SERPINH), and **D**, matrix metalloproteinase 9 (MMP9) in juvenile rainbow trout fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (6.4, 15.8 and 47.8 µg Se/g d.m.) for 60 d. Data are mean ± S.E.M of n = 5 pooled hearts (3 hearts/sample) per group. Transcript abundance was determined by quantitative RT-PCR. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

3.4.4 Energy storage and key metabolic enzyme mRNA transcript abundance

Skeletal muscle and liver are two tissues where glycogen and triglycerides are important metabolic stores in fish and these organs possess intermittently high metabolic rates. In contrast, although the heart is not a major body storage site for glycogen or lipid in fish, these two energy stores are key energy substrates for this important organ that has continuous high metabolic demand. Although glycogen and triglyceride concentrations did not statistically differ in skeletal muscle and heart tissues, liver concentrations for both energy storage molecules did. Fish fed 15.8 and 47.8 μg Se/g d.m. for 60 d had significantly greater hepatic glycogen (46 ± 2.8 and 69 ± 6.8 mg/g, respectively) and triglyceride (1.1 ± 0.1 and 1.3 ± 0.1 mg/g, respectively) concentrations when compared to the controls (33 ± 3.3 and 0.6 ± 0.1 mg/g, respectively) ($p < 0.05$, Figure 3.6 A, B).

Key regulatory genes associated with energy homeostasis (β-hydroxyacyl coenzyme A dehydrogenase [HOAD] and citrate synthase [CS]), were quantitated in heart, liver and skeletal muscle using qPCR. A significant decrease in liver CS transcript abundance was observed in the 15.8 and 47.8 μg Se/g d.m. exposure group when compared to the control ($p < 0.05$, Figure 3.6 C). In contrast, CS transcript abundance in skeletal muscle from the highest Se group (47 μg Se/g d.m.) was significantly increased compared to control, while no significant change among groups in CS abundance was noted in cardiac tissue (Figure 3.6 C). Similarly, HOAD mRNA transcript abundance was significantly reduced in 6.4 μg Se/g d.m. skeletal muscle ($p < 0.05$; Figure 3.6 D), but unchanged in cardiac muscle. Conversely, HOAD mRNA was significantly increased in liver of the 47.8 μg Se/g d.m. exposed groups when compared to the control ($p < 0.05$; Figure 3.6 D).

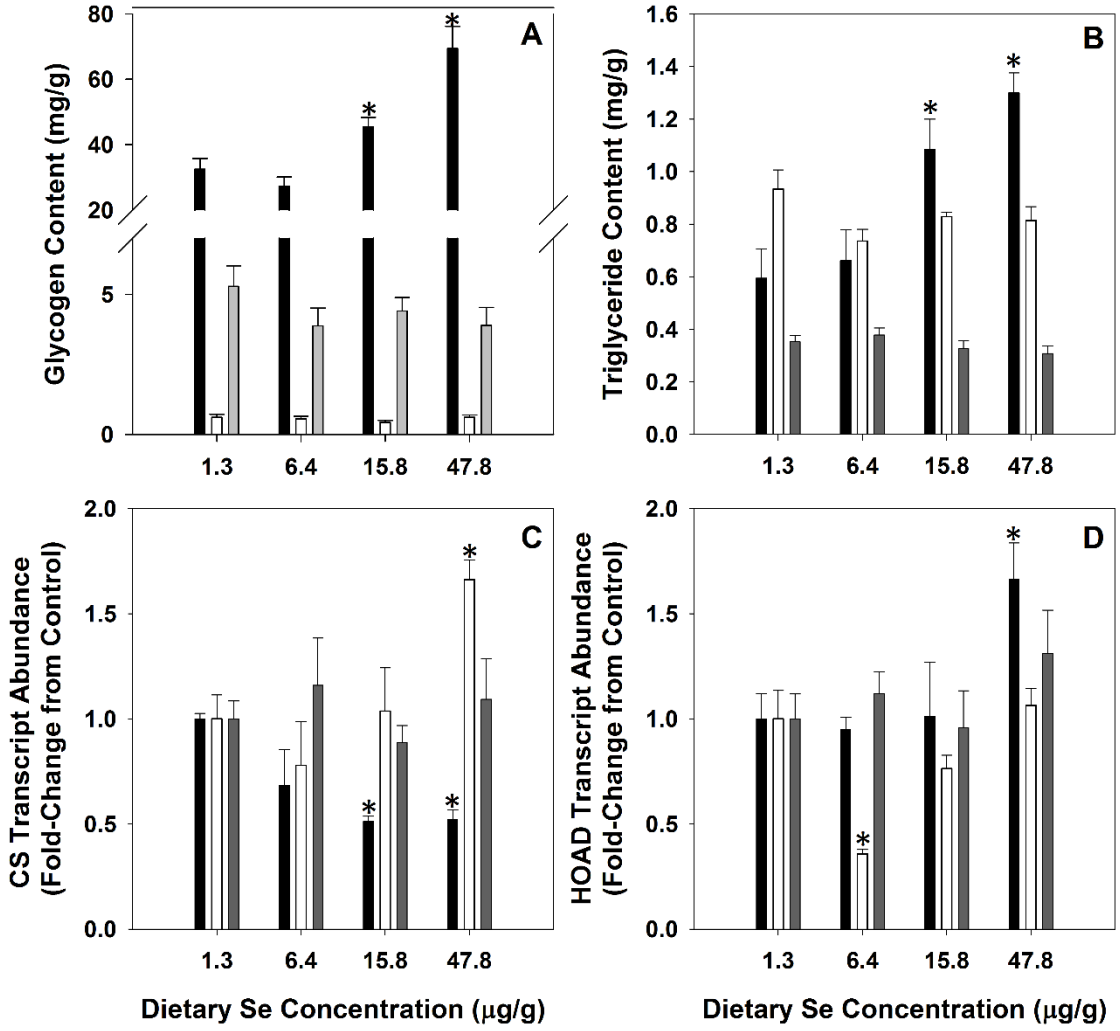

Figure 3.6. Energy stores and metabolic enzymes in rainbow trout tissues after 60-d dietary exposure to selenomethionine. **A**, glycogen concentrations and **B**, triglyceride concentrations. Data are mean ± S.E.M of n = 10 fish/group. mRNA abundance of **C**, citrate synthase (CS) and **D,** β-hydroxyacyl coenzyme A dehydrogenase (HOAD) in liver (black bars), skeletal muscle (white bars) and heart (gray bars) of juvenile rainbow trout fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (6.4, 15.8 and 47.8 µg Se/g d.m.) for 60 d. Transcript abundance was determined by quantitative RT-PCR. Data are mean ± S.E.M of n = 5 fish/group for liver and muscle and n = 5 pooled hearts (3

hearts/sample) per group. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

3.4.5 Gene expression of antioxidant enzymes

Gene expression of selected oxidative stress (glutathione peroxidase 1A [GPX1A] glutathione-S-transferase pi class [GST-pi], superoxide dismutase [SOD] and catalase [CAT]) were monitored in liver, skeletal muscle and heart tissues using qPCR. Significant increases in GST-pi mRNA abundance were observed in skeletal muscle and liver, but instead decreased in heart tissue in the highest exposed (47.8 µg Se/g d.m.) group when compared to the control ($p < 0.05$; Figure 3.7 A). Interestingly, significant increases of GPX1A mRNA abundance were observed in skeletal muscle and liver, while significant decreases in abundance were observed in heart tissues in the 6.4 and 47.8 µg Se/g d.m. treatment group compared to the control ($p < 0.05$; Figure 3.7 B). Significant decreases in SOD mRNA transcript abundance were observed in all three tissues in the 6.4 µg Se/g d.m. treatment group, but only in liver and skeletal muscle in the 15.8 µg Se/g d.m. group when compared to the control ($p < 0.05$; Figure 3.7 C). Additionally, in liver, but not other tissues, a significant increase in CAT mRNA transcript abundance was observed in the 47.8 µg Se/g d.m. treatment group, when compared to the control ($p < 0.05$; Figure 3.7 D).

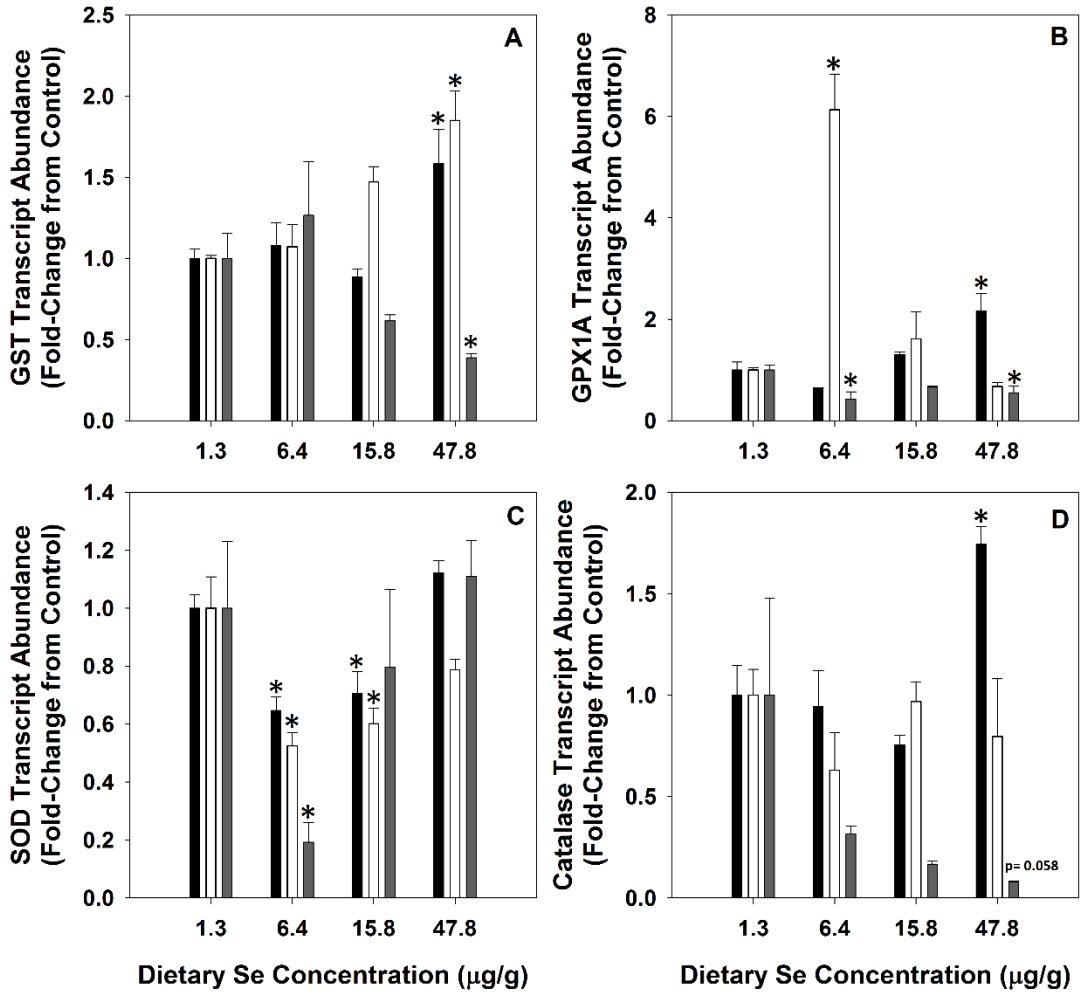

Figure 3.7. mRNA abundance of **A**, glutathione-s-transferase pi class (GST-pi), **B**, glutathione peroxidase 1a (GPX1A), **C**, superoxide dismutase (SOD), and **D**, catalase (CAT) in liver (black bars), skeletal muscle (white bars) and heart (gray bars) of juvenile rainbow trout fed control (1.3 µg Se/g d.m.) or selenomethionine spiked diets (6.4, 15.8 and 47.8 µg Se/g d.m.) for 60 d. Data are mean ± S.E.M of n = 5 liver and muscle, and n = 5 pooled hearts (3 hearts/sample) per group. Transcript abundance was determined by

quantitative RT-PCR. * Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$).

3.4.6 Glucose Tolerance Test, methylglyoxal levels, cataracts and behavior

After 24 hr fasting, baseline (pre-glucose challenge) blood glucose levels did not differ significantly among groups, ranging from 3.1-3.8 mmol/L (Table 3.2). Peak blood glucose ranged from 17-22 mmol/L, and did not differ significantly among groups (Table 3.2), with time to peak glucose being significantly longer in the highest 47.8 µg Se/g d.m. treatment at 44.0 ± 4.0 h compared to the control 3.0 ± 0.0 h (Table 3.2). At 48 hr following intraperitoneal injection of glucose, all treatments had blood glucose that had returned to their baseline levels, except for the 47.8 µg Se/g d.m. treatment, which remained significantly higher ($p < 0.05$) than the control, at 19.8 ± 0.6 mmol/L (Table 3.2). This prolonged hyperglycemia at 48 hr in the highest Se group led to significantly increased plasma levels of the toxic glucose metabolite, methylglyoxal, compared to control (Table 3.3). In addition, although only qualitative observations could be made, there was a noticeable increase in the incidence of cataracts (Table 3.3). Incidence went from zero in the control group to 8% and 36% in the 15.8 and 47.8 µg Se/g d.m. treatment groups. Furthermore, ad hoc behaviour testing was conducted after noting this increasing incidence of cataracts in Se-exposed fish. This behaviour test assessed how long it took trout to find their food during feeding time. Qualitatively, fish in the highest dietary Se group took longer to find their food compared to control (Table 3.3).

Table 3.2. Glucose tolerance test in fish fed either a control or selenomethionine-spiked diet for 60 d. Rainbow trout were injected intraperitoneal with 0.5 g glucose/kg body weight. Data are mean ± S.E.M. of n = 6 samples.

Treatment [Se] (µg/g dry mass)	Fasting Blood Glucose (mmol/L)	Peak Blood Glucose (mmol/L)	48hr Blood Glucose (mmol/L)	Time to Peak (hr)
1.3	3.6 ± 0.1	21.0 ± 0.9	5.4 ± 0.4	3.0 ± 0.0
6.4	3.5 ± 0.1	22.0 ± 0.7	5.3 ± 0.7	9.5 ± 3.4
15.8	3.1 ± 0.2	17.3 ± 1.2	7.6 ± 0.3	9.0 ± 1.3
47.8	3.8 ± 0.4	19.8 ± 0.6	19.8 ± 0.6*	44.0 ± 4.0*

*, Significantly different from the control group using one-way ANOVA followed by Fisher LSD post-hoc test ($p < 0.05$).

Table 3.3. After 60 d of dietary exposure to 6.4, 15.8 and 47.8 µg Se/g d.m., rainbow trout plasma methylglyoxal at 48hr after glucose challenge (n = 4), cataract prevalence (%), and feeding behaviour as a surrogate for visual function are shown. The behaviour test measured the time taken for all fish in a given tank to return to normal swimming behaviour after feeding a standard amount of feed (67.5 g). Data are mean ± S.E.M.

Treatment [Se] (µg/g dry mass)	Plasma methylglyoxal (µM)	Cataract prevalence (%)	Time to return to normal behaviour after feeding (s)
1.3	0.76 ± 0.23	0	3.8
6.4	ND	0	3.9
15.8	ND	8	4.0
47.8	2.39 ± 0.89*	36	5.1

* Significantly different from control group using one-way ANOVA followed by Fisher LSD post hoc test ($p < 0.05$), ND - not determined.

3.5 Discussion

Selenium has proven to be a contaminant of growing concern. It is well known that oviparous species are especially vulnerable as maternal transfer is the major route of Se exposure to eggs and embryo-larval fishes (Lemly, 1997, 2002; Janz et al., 2010). To our knowledge, this is the first study to investigate the cardiovascular and glycemic control effects of chronic dietary Se exposure in juvenile rainbow trout. The most prominent findings of this study were that environmentally relevant Se-Met exposure can disrupt normal cardiovascular function, impair energy homeostasis and promote development of glucose intolerance in juvenile rainbow trout. The cardiac effects appeared adaptive and included increased stroke volume (the volume of blood pumped in each individual cardiac contraction), increased ejection fraction (the percentage of blood that is ejected out of the ventricles with each contraction), and most notably, an increase in cardiac output (the volume of blood pumped per minute). In addition, juvenile trout had significantly greater liver stores of triglycerides and glycogen, but proved to be unable to use these stores, regulate glycemic responses or prevent glucose intolerance complications.

3.5.1 Se dosing and kinetics

Se diets chosen were considered environmentally relevant, and were modeled after previous studies eliciting sublethal toxicities in adult fish (Thomas et al., 2013; McPhee and Janz, 2014; Pettem et al., 2017). In addition, similar Se concentrations have been observed in fish collected from Se impacted sites (Lemly, 1997; Fan et al., 2002; Hamilton, 2004; Muscatello et al., 2006; Muscatello and Janz, 2009). Muscle Se concentrations increased in a dose-dependent manner and were directly proportional to

concentrations of Se in the diet. Muscle Se in both the 15.8 and 47.8 μg Se/g d.m. exposure groups had significantly greater concentrations when compared to the control following 14 d of dietary exposure. Muscle Se concentrations continued to increase, as bioaccumulation was readily apparent, peaking at 74 ± 4 μg Se/g d.m. in the highest exposure group after 56 d of dietary exposure. These levels are far above the 2016 USEPA muscle tissue guideline levels of 11.3 μg Se/g (USEPA, 2016). These results seem to indicate that at the lowest dose of 6.4 μg Se/g d.m., the fish are able to regulate, and effectively excrete excess Se, as muscle concentrations stayed relatively constant during the 60 d exposure. However, at greater concentrations, Se overrides the physiological ability to excrete excess Se and it begins to bioaccumulate. Previous Se-Met studies reported similar concentrations in whole-body Se assimilation in zebrafish, cutthroat trout (Oncorhynchus clarkii), white sturgeon (Acipenser transmontanus) and bluegill (Lepomis macrochirus) (Cleveland et al., 1993; Linville et al., 2006; Thomas and Janz, 2011; Pettem et al., 2017). There were no significant effects of dietary Se-Met exposure on total length, mortality, cardiosomatic index (CSI) or condition factor, however there was a significant decrease in body weight in the lowest exposure group of 6.4 μg Se/g d.m. and a significant increase in hepatosomatic index (HSI) in the 47.8 μg Se/g d.m. exposure group compared to control.

Surprisingly, dietary Se-Met exposure significantly enhanced normal cardiac function in juvenile rainbow trout. The results of the current study contrast trends seen in previous studies examining exposure to petroleum contaminants and polycyclic aromatic hydrocarbons, where bradycardia and arrhythmias characteristic of AV blockade were observed in both zebrafish (Incardona et al., 2004; Zhang et al., 2013) and pacific herring

(Clupea pallasii) embryos (Incardona et al., 2009). More importantly, the findings in this current study in trout are opposite to results found in adult zebrafish using similar dietary Se-Met concentrations and exposure duration (Pettem et al., 2017). Whether differences are due to species sensitivity differences or warm versus cold-water fish is debatable. Chapman (2007) argued that cold-water fish species, including white sucker (Catostomus commersoni), northern pike (Esox lucius), and trout (cutthroat, brook, rainbow trout) have higher tolerances to Se taken up via dietary sources than warm-water fish species. However, since opposite results, not just a difference in sensitivity were observed, this may instead indicate a fundamental species difference between zebrafish and trout in their cardiac response to Se-Met exposure. While the cardiotoxic response in zebrafish was maladaptive (e.g. decreased cardiac output), the results of the present study in trout were instead highly adaptive since trout in the highest exposure group had greater stroke volume, ejection fraction and cardiac output. This would result in greater blood being delivered to the gills, thus increasing the amount of oxygen and nutrients being delivered to tissues (Olson and Farrell, 2006). This result of increased cardiac output is consistent with the expected physiological response to an increase in metabolic demand (MacKinnon and Farrell, 1992). Previous studies from our group exposed zebrafish to similar Se-Met concentrations and observed significant increases in oxygen consumption (MO2), and reduced swim performances (Ucrit), leading to reduced aerobic capacity (Thomas and Janz, 2011; Thomas et al., 2013). While we do not know whether rainbow trout oxygen consumption was similarly increased since the trout were too big for our swim tunnel respirometry equipment, we can say that the observed cardiac response would be consistent if metabolic demand were increased after Se-Met exposure in trout.

The increased cardiac output observed in the current study in rainbow trout exposed to the highest dietary level of Se-Met was not due to a change in heart rate (in fact atrial rate significantly decreased, while ventricular rate was unchanged). While the ratio of atrial to ventricular rates was not significantly different among groups, no clear trend in cardiac hERG expression was noted, with a significant increase in mRNA transcript abundance noted only in the lowest Se-Met exposure group where no functional changes were observed. Lower hERG expression has been reported to cause impairment of conduction through the atrioventricular node in fish (Langheinrich et al., 2003; Brette et al., 2017), but the effects of a higher hERG expression is unclear.

The increased cardiac output after exposure to the highest dietary Se-Met instead arose from an increase in stroke volume in the current study. While neither EDV nor ESV were significantly affected by treatment, there was a non-significant trend for EDV to increase suggestive of better diastolic filling. A significant increase in SERCA heart mRNA transcript abundance was observed in the highest Se-Met group in this study. The SERCA gene is crucial for relaxation of cardiac muscle by removing intracellular calcium to facilitate diastolic relaxation (Korajoki and Vornanen, 2012; Keen et al., 2017). Thus increased SERCA expression would allow the heart to resequester calcium and relax faster, leading to longer periods in diastole, consistent with improved diastolic filling (Keen et al., 2017). Further support for improved diastolic filling comes from the observation in this study of a significant increase in passive filling of the ventricle (increased passive AV Doppler velocity and increased passive/active AV velocity ratio). This led to the hypothesis that high dietary Se-Met was causing the ventricle to become more compliant.

The hypothesis was supported by the observed significant 3-fold increase in MMP9 and decrease in SERPINH heart mRNA transcript abundance in the highest 47.8 μg Se/g d.m. exposure group. MMPs are a class of proteolytic enzymes that have important vascular and cardiac remodeling properties (Seliktar et al., 2001; Cawston and Young, 2010). In contrast, SERPINH is a stress inducible protein in the endoplasmic reticulum that plays a major role as a molecular chaperone and is essential for the post-translational folding of fibril-forming collagens (Rocnik et al., 2002). While increases in SERPINH concentrations are associated with increases in extracellular matrix formation, which can lead to an increase in fibrotic diseases in the mammalian heart (Rocnik et al., 2002), the observed decrease in cardiac SERPINH would decrease collagen content. Concurrent with increased collagen turnover through increased MMP9, the results of the current study strongly suggest the rainbow trout heart responds to high dietary Se-Met by decreasing ventricular compliance, promoting increased diastolic filling and improving cardiac output. These series of findings are in direct contrast to that of a previous study from our group using similar Se-Met concentrations in zebrafish, where a significant decrease in MMP2 was observed, leading to an increase in ventricular fibrosis and decreased cardiac output (Pettem et al., 2017). The reported anti-fibrotic cardiac changes observed in the current study in rainbow trout exposed to high dietary Se-Met resemble what has been reported previously in response to warm temperature acclimation in the same species (Keen et al., 2017). Of note is the fact that zebrafish cardiac remodeling in response to thermal acclimation is opposite to that of rainbow trout (Keen et al., 2017), confirming the tendency for opposite cardiac remodeling responses between these two species in response to different stimuli.

3.5.2 Alterations in energy stores and intermediary metabolic enzymes

While altered cardiac function was suggestive of increased metabolic demand in rainbow trout exposed to high dietary Se-Met, hepatic levels of stored energy (triglycerides and glycogen) were instead elevated in trout fed ≥15.8 μg Se/g d.m. This threshold to cause increased triglyceride and glycogen in Se-Met-exposed rainbow trout is similar to that observed in previous adult zebrafish studies (Thomas and Janz, 2011; Thomas et al., 2013; McPhee and Janz, 2014; Pettem et al., 2017) and various small-bodied fish species collected from Se impacted sites (Bennett and Janz, 2007; Kelly and Janz, 2008; Goertzen et al., 2012). In contrast to previous studies where energy storage molecules in whole body homogenate were examined, trout were large enough to measure individual organ levels of triglycerides and glycogen. While liver stores were elevated, there were no significant differences in skeletal or cardiac muscle energy stores among treatments. Whether the increased energy stores in liver after Se-Met exposure in the current rainbow trout study was sufficient to produce an increase in whole body stores is not clear since whole body homogenate was not measured in this study. Alternatively, it may be the effect of excess dietary Se-Met has an effect restricted to just a single organ unlike small-bodied fish in our previous studies where a more wide-spread increase in energy stores in multiple organs led to increased levels in whole body homogenate. Future studies should address this issue.

In order to elucidate mechanisms of metabolic dysfunction, mRNA expression of key aerobic energy metabolizing enzymes, citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD) were measured. HOAD is an important mitochondrial enzyme involved in β-oxidation of fatty acids, whereas CS is a key enzyme

involved in the citric acid cycle and is used as an index of aerobic metabolic activity (Rajotte and Couture, 2002). Previous studies have identified altered liver and whole-body enzyme activities of both CS and HOAD in yellow perch (Perca flavenscens), fathead minnow (Pimephales promelas), and spottail shiner (Notropis hudsonius) after exposure to environmental pollutant stressors (Rajotte and Couture 2002; Goertzen et al., 2011, 2012). In the present study, elevated dietary Se-Met exposure resulted in significant down-regulation of liver CS mRNA transcript abundance from trout exposed to ≥15.8 μg Se/g d.m. This decrease in mRNA would be expected to decrease aerobic metabolic activity, providing a potential explanation for the observed significant increase in hepatic glycogen and triglycerides in our Se-Met exposed fish. In contrast, the highest Se-Met exposure group had greater CS mRNA transcript abundance in skeletal muscle tissue, while no change in cardiac CS abundance was noted. A significant up-regulation of liver HOAD mRNA transcript abundance was also observed in the highest Se-Met exposed treatment. If HOAD mRNA was translated proportionately to HOAD protein in liver, an increase in fatty acid oxidation and depletion of hepatic triglyceride stores would be expected, but instead the opposite effect was observed. In skeletal muscle, a decreased mRNA transcript abundance of HOAD was observed in the 6.4 μg Se/g d.m. group, an effect expected to increase triglyceride content. However, the triglyceride levels were unchanged by treatment in skeletal muscle. Taken together, changes in mRNA expression of hepatic CS may correlate to the observed increases in hepatic glycogen and triglyceride content. Otherwise, changes in HOAD expression in all tissues or changes in CS expression in skeletal muscle are inconsistent with the lack of change in glycogen and triglyceride levels observed in these other tissues. The accumulation of fatty acids

specifically in the liver after high dietary exposure to Se agrees with a previous rat study using 6 weeks of daily oral selenite administration, an effect associated with impaired glucose handling (Wang et al., 2014).

3.5.3 Glucose tolerance, methylglyoxal, oxidative stress, cataracts and behavioural alterations

To determine if excess Se was causing glucose intolerance in the rainbow trout in this study, an intraperitoneal glucose tolerance test (GTT; standard clinical test for diabetes in mammals) was performed. The failure of blood glucose to return to baseline at 48 hr after glucose challenge in rainbow trout fed 47.8 μg Se/g d.m. for 60 d indicates that this level of Se-Met exposure produced glucose intolerant fish. While past literature indicates that Se deficiency may contribute to the progression of type 2 diabetes (T2D) in mammals (Kljai and Runje, 2001), with adequate Se supplementation, the risk of T2D is diminished (Park et al., 2012). However, increasingly both rodent and human studies suggest an excess of Se may similarly produce a T2D-like state (Rasekh et al., 1991; McClung et al., 2004; Stranges et al., 2007; Bleys et al., 2007; Laclaustra et al., 2010; Wang et al., 2008; Mueller et al., 2008; Mueller et al., 2009; Stranges et al., 2010, 2011; Labunsky et al., 2011; Zeng et al., 2012; Liu et al., 2012; Wang et al., 2014), although no literature exists for fish.

Some reactive oxygen species are required for proper insulin signaling, but an imbalance caused by excess Se toward a more pro-oxidant oxidative stress state is suggested to mediate this change in insulin sensitivity, with selenoprotein P and glutathione peroxidase inactivation by ROS being key triggers (Steinbrenner, 2013; Wang et al., 2014). In the current study, mRNA transcript abundance of heart GST-pi class and

GPX1A were significantly down-regulated, while up-regulated in both liver and skeletal muscle of the highest exposure group. Since catabolism of Se-Met has been reported to cause oxidative stress in fish (Palace et al., 2004), this suggests the heart may have been particularly vulnerable to increased damage from ROS (Xia et al., 2004; Musaro et al., 2010). A previous study reported a link between the potential for ROS to influence extracellular matrix remodelling through the activation of MMPs (Spinale, 2002). In the normal healthy myocardium, MMPs are expressed at very low levels but are substantially upregulated in congestive heart failure (Spinale, 2002). ROS can change overall collagen and fibroblast synthesis, but can also increase MMP expression (Spinale, 2002), and treatment using ROS scavengers can help reduce detrimental cardiac remodelling in mammals (Byrne et al., 2003).

Moreover, hyperglycemia is known in mammals to cause excess production of the toxic glucose metabolite, methylglyoxal (Desai et al., 2010; Adolphe et al., 2012; Kalapos, 2013). To our knowledge, this is the first report that methylglyoxal is not only measurable in any fish species, but also that increased levels are associated with glucose intolerance, similar to that reported in human diabetics (Desai et al., 2010; Kalapos, 2013). Methylglyoxal, being a reactive carbonyl, also increases oxidative stress as well as covalently modifying macromolecules to cause dysfunction and acting as a precursor for advanced glycation end products that cause cataracts in humans (Desai et al., 2010; Kalapos, 2013). The observation that trout in the highest Se-Met exposure group developed cataracts agrees well with previous studies reporting cataracts in rats exposed to selenite (Shearer et al., 1983) or fish from a high Se environment at Belews Lake, North Carolina (Lemly, 2002). Selenium has been shown through confocal X-ray

synchrotron-based fluorescent imaging to preferentially accumulate in the eye lens in zebrafish (Choudhury et al., 2015). In addition, adult zebrafish vision has been investigated using behavioural assays, and adults fed with Se-Met spiked diets exhibited reduced escape responses and their F1 progeny had smaller eyes and fewer positive responses in phototaxis, oculomotor and optokinetic response assays (Raine et al., 2016). Glutathione peroxidases (GPX1 and GPX3; both selenoproteins) found in the eye (Pirie, 1965) normally protect the lens from oxidative damage (Flohe, 2005), but a previous study reported excess Se caused decreased anti-oxidants and increased oxidative damage in the eye (Combs and Combs, 1986). The relevance of cataracts with excess Se-Met is supported by our qualitative observation of prolonged feeding behaviour. Trout exposed to high dietary Se-Met clearly were having difficulties finding their food, which could potentially lead to decreased survival in wild fish exposed to similar selenium levels.

3.6 Conclusion

In conclusion, the present study was able to demonstrate that chronic exposure to environmentally relevant concentrations of dietary Se-Met can alter overall cardiovascular function, impair energy homeostasis, cause glucose intolerance and hyperglycemia, increase levels of the toxic glucose metabolite, methylglyoxal, and cause cataracts that lead to behavioural changes in juvenile rainbow trout. Se-Met exposure caused significant increases in stroke volume, ejection fraction and cardiac output, consistent with decreased cardiac compliance and an adaptive physiological response in the trout heart. In addition to the direct physiological effects, Se-Met produced effects at the cellular level, causing up-regulation of key cardiac remodeling genes (MMP9), and down-regulation of fibril forming collagens (SERPINH) and metabolic (CS) gene

expression. Based on the results of this study, a proposed adverse outcome pathway is summarized in Figure 3.8. Overall, these effects would alter the aerobic capacity of fish, impair their ability to find food and have potential ecological consequences on overall fitness.

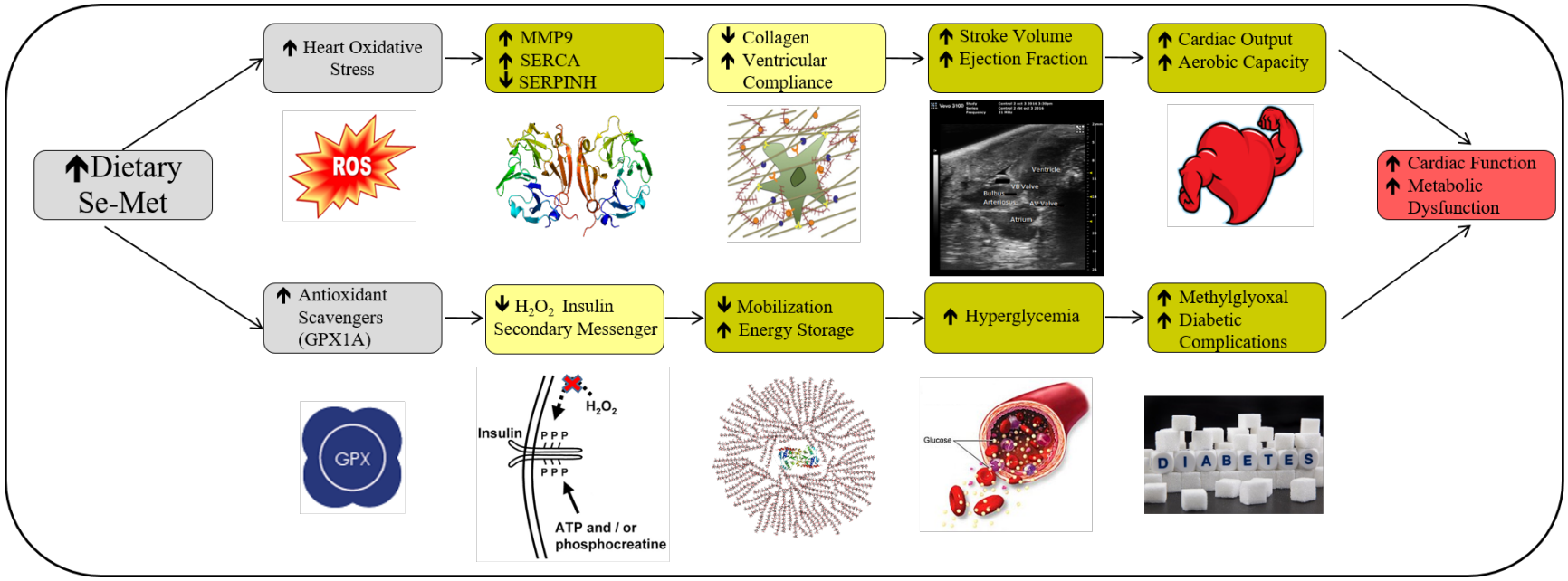

Figure 3.8. Hypothesized adverse outcome pathway (AOP) linking dietary selenomethionine exposure with increased cardiac function and adverse metabolic toxicity. Lighter, yellow coloured boxes represent changes that are assumed, but were not directly measured.

CHAPTER 4

4.0 GENERAL DISCUSSION

4.1 Project Rationale and Summary

Selenium has proven to be a contaminant of growing concern. It has been shown in numerous field and laboratory experiments to cause different toxic effects to not only fish, but also birds and amphibians. Although there is potential for aqueous Se to cause toxicity, in Se-contaminated aquatic environments diet has proven to be the major route of exposure. In addition, the maternal transfer of Se to eggs and embryo-larval fish through vitellogenesis and yolk sac resorption has been shown to cause F1 generational toxicities. Because of this, not only are adults and developing embryos at risk, but future offspring as well. This can cause a cascade of effects, potentially leading to an entire collapse of a population or even ecosystem. The adverse effects that high Se exposures can have on species health are not only well documented in laboratory experiments, but are also evident in numerous case studies, such as the Kesterson disaster and the Belews Lake tragedy. In the mid-80s, thousands of birds and fish were found severely deformed or dead in the Kesterson National Wildlife Refuge, following large-scale bioaccumulation of Se from agricultural runoff (Presser, 1994). This had huge environmental ramifications, including the abandonment of 100,000 acres of once irrigated farmland in the San Joaquin Valley (Moore et al., 1990). Belews Lake, North Carolina was severely contaminated with Se by wastewater from a coal-fired power upstream in the 1970s and 20 resident fish communities were tracked for over two decades. Among the consequences seen due to chronic Se poisoning were huge numbers of dead or deformed fish and corneal cataracts (Lemly, 2002). Replicating dietary

exposures to organic Se in the form of Se-Met in laboratory experiments has been demonstrated to cause greater mortality and deformities, impair the physiological stress response, alter swimming performance, aerobic metabolism, and cause metabolic dysfunction (Teh et al., 2004; Tashjian et al., 2006; Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013). Although the toxic effects of dietary Se-Met exposure are well known, there is a lack of laboratory research investigating the potential cardiovascular effects of elevated Se exposure. In addition, while the majority of knowledge surrounding Se toxicity stems from juvenile and larval fish studies, less is known surrounding the overall toxic effects to adult fish species. Therefore, the overall objective of this thesis was to further characterize the sub-lethal effects of chronic dietary Se-Met exposure, by investigating the potential cardiovascular and metabolic consequences in both adult zebrafish and rainbow trout.

The most notable findings of this study were that environmentally relevant dietary Se-Met exposure can disrupt normal cardiovascular function and impair energy homeostasis in both adult zebrafish and juvenile rainbow trout. However, the cardiovascular effects seen varied substantially between species. While Se-induced cardiotoxicity was seen in zebrafish with reduced stroke volume, ventricular contractile rate, and most prominently a decrease in cardiac output, the opposite trend was seen in juvenile (yearling) rainbow trout exposed to similar concentrations of dietary Se-Met. Rainbow trout had increased stroke volume, increased ejection fraction, and an increase in cardiac output. The failure of blood glucose to return to baseline at 48 hr after glucose challenge in rainbow trout fed 47.8 μg Se/g d.m. for 60 d indicates that this level of Se-

Met exposure produced glucose intolerant fish. Both fish also experienced significant alterations in bioenergetic status and variations at the mRNA cellular level.

4.2 Comparison Between Adult Zebrafish and Juvenile Rainbow Trout Dietary Se-Met Exposures

4.2.1 Selenium concentrations

The majority of Se exposure occurs through the diet via its organic form, Se-Met, which easily bioaccumulates in aquatic food webs (Fan et al., 2002). The ease for Se to bioaccumulate at the base of the food web is an area of concern as dietary organoselenium progresses through consumers at higher trophic levels, who integrate these seleno-amino acids into proteins, resulting in high concentrations of Se in upper predatory species, including fish (Furr et al., 1979; Hamilton, 2004). Fishes previously collected from Se contaminated sites had upwards of 80% of total Se in the form of Se-Met (Phibbs et al., 2011; Franz, 2012). Therefore, for the purpose of my thesis I spiked Se-Met into commercially available fish food, and chronically exposed both rainbow trout and zebrafish.

In both experiments, Se concentrations were significantly greater than the control diets and were observed to cause a downstream cascade of effects, although slightly different between species. Commercially available flake food and trout pellets were spiked with Se-Met for both the zebrafish and rainbow trout experiments, respectively. The control diets were made by replicating the spiking methods, without the addition of Se-Met and their concentrations were both minimal and deemed acceptable to be background control levels (1.1 and 1.3 μg Se/g d.m, respectively). Toxicity has been shown to occur at concentrations exceeding 3 μg/g d.m. in fishes (Janz, 2012), therefore

these concentrations can be considered Se-normal. Zebrafish morphometrics did not change in relation to diet, but whole body Se concentrations increased in a dose dependent manner. This however did not come as a surprise. Due to the structural similarity between Se-Met and the amino acid methionine, Se-Met can avoid biotransformation and be directly integrated into any methionine-containing protein (Suzuki and Ogra, 2002). Because of this, we see in a dose-dependent manner increasing concentrations of Se-Met in tissues with high rates of protein synthesis including the liver, kidney, skeletal muscle, gonads as well as erythrocytes (Schrauzer, 2000). This was also seen in the rainbow trout, as muscle Se concentrations steadily rose over the 60 day exposure period, clearly showing the ability for bioaccumulation. In rainbow trout, a significant decrease in body weight in the 6.4 μg/g d.m. treatment group, a significant increase in hepatosomatic index, and a large number of fully developed cataracts in the highest 47.8 μg/g d.m group were observed, but no other morphometric differences were observed. There are varying results on morphometric changes in response to dietary Se-Met exposure, including increased mass and length in juvenile rainbow trout, no changes between treatments in zebrafish, and decreased mass and length in juvenile Sacramento splittail (Teh et al., 2004; Vidal et al., 2005; Thomas et al., 2013). Morphometrics are therefore not a reliable sub-lethal endpoint to compare in dietary Se-Met exposures. Following 90 day exposure, whole body zebrafish Se concentrations ranged from 0.7 to 9.2 μg/g d.m. and rainbow trout muscle Se concentrations following 60 day exposure ranged from 2.9 to 74.3 μg/g d.m. These concentrations were both above the 2016 United States Environmental Protection Agency (USEPA) muscle tissue criterion of 11.3 μg Se/g d.m. (USEPA, 2016). Aqueous Se was measured to make sure levels were not

affecting the overall route of exposure, and concentrations were found to be negligible between treatments, ranging from 0.40 μg/L in the control tank to 0.42 μg/L in the highest exposure group. This reinforced that any experimental results seen can solely be based on ranging spiked Se-Met diets.

4.2.2 Cardiovascular implications

Cardiac function was significantly affected by chronic dietary Se-Met exposure in both adult zebrafish and juvenile (yearling) rainbow trout, although with varying results. In the first experiment, the most prominent finding was that exposure to environmentally relevant concentrations of dietary Se-Met significantly reduced stroke volume, ventricular contractile rate and cardiac output. These results follow trends seen in previous polycyclic aromatic hydrocarbon (PAH) exposure studies, where bradycardia and arrhythmias characteristic of AV blockade were observed in both zebrafish (Incardona et al., 2004; Zhang et al., 2013) and pacific herring (*Clupea pallasii*) embryos (Incardona et al., 2009). In addition, studies using zebrafish embryos have shown altered Ca^{2+} handling in the heart, causing ventricular arrhythmias following the exposure to PAHs (Zhang et al., 2013; Gerger et al., 2015). This however, was one of the first studies to non-invasively look at the cardiotoxic effects of chronic Se exposure to adult fish *in vivo*. Previous studies exposed zebrafish to similar dietary Se-Met concentrations and observed significant increases in oxygen consumption (MO_2), and reduced swim performance (U_{crit}), which were interpreted as reduced aerobic capacity (Thomas and Janz, 2011; Thomas et al., 2013). Cardiac activity is normally regulated in response to changes in oxygen supply and demand. Thus, greater cardiac output would be predicted in the elevated Se-Met exposed zebrafish based on higher oxygen demand, whereas we

instead observed reduced cardiac output in the 28.8 µg Se/g d.m. treatment. The underlying reasons behind this cardiotoxicity may have been attributed to the ventricular fibrosis observed. The significant increase in echodensity at the junction between the atrium and ventricle surrounding the AV valve, coupled with the significant down-regulation of heart MMP2 mRNA transcript abundance, could lead to increased fibrosis or stiffening at the AV valve. These taken together could have led to the cardiotoxicity observed.

Interestingly, in the second experiment using juvenile (yearling) rainbow trout, this impaired cardiovascular response was not seen with comparable study conditions. Although this study design had a shorter exposure period (60 days), the Se-Met concentrations were slightly greater. The most notable findings of this study were that rainbow trout exposed to dietary Se-Met had significantly increased stroke volume, ejection fraction and cardiac output. These results are almost directly opposite to those seen in the previous zebrafish exposure. It is known that zebrafish are one of the most sensitive fish species to Se exposure, but it is perplexing to see just how varying these results were between species. Whether this is due to species sensitivity differences or warm vs cold water fish is unclear. As mentioned in chapter 3, Chapman (2007) provides evidence that cold-water fish species, including northern pike (*Esox Lucius*), white sucker (*Catostomus commersonii*), and trout [rainbow (*Oncorhynchus mykiss*), cutthroat (*Oncorhynchus clarkia*), brook (*Salvelinus fontinalis*)] have greater tolerance to dietary Se than warm-water fish species. This means that at higher Se concentrations, similar cardiotoxicity results as previously seen in zebrafish could have been observed. However, this emphasizes the narrow margin between essentiality and toxicity with Se, as the

present study could argue that Se supplementation was considered beneficial to the cardiovascular system, as juvenile rainbow trout in the highest exposure group had greater stroke volume, ejection fraction and cardiac output. This would result in greater blood being ejected, thus increasing the amount of oxygen and nutrients being delivered to tissues (Olson and Farrell, 2006), compared to the results seen in the zebrafish experiment which would have resulted in insufficient oxygen and nutrients being delivered to tissues, causing further physiological impairment. In addition, no ventricular fibrosis was observed as was seen in the zebrafish experiment. This could have been attributed to the observed increase in cardiac MMP9 and decrease in cardiac SERPINH mRNA transcript abundance. At the cellular level, this anti-fibrotic response could help protect the heart from laying down collagen deposits around the valve. These deposits can cause narrowing at the opening of the valve and would lead to inadequate blood movement through the chambers and a systemic reduction in overall blood flow. However, due to the anti-fibrotic response seen in this experiment, the valves may be protected and perhaps even considered improved. Furthermore, a significant increase in SERCA heart mRNA transcript abundance was seen. This gene is crucial for relaxation and contraction of cardiac muscle, triggering and sustaining calcium propagation waves (Korajoki and Vornanen, 2012). Perhaps due to this protection against fibrosis, there was an observed increase in stroke volume and ejection fraction in the highest exposure treatment. This would mean a greater volume of blood is being ejected with each individual heartbeat, allowing for greater delivery of oxygen and nutrients to tissues. This increase in ejection fraction would mean the heart is actually working more effectively when compared to the control fish. Whether this is an adaptive response due to the

increase in oxygen consumption (MO_2), as seen in previous zebrafish studies (Thomas and Janz, 2011; Thomas et al., 2013), or if excess Se concentrations may be considered beneficial to the cardiovascular system is unclear, and further studies are warranted.

The differences seen here could be attributed to relative species sensitivity or it could be as simple as the differences in exposure temperature. Zebrafish, which were reared at 28°C, are known to be one of the most sensitive fish species to Se toxicity with egg Se larval deformity toxicity thresholds (EC10s) of 7 µg Se/g d.m. (Thomas and Janz., 2015). This is compared to the 12°C reared rainbow trout, which have a reported EC10 value of 21.1 (Holm et al., 2005). As is known, the solubility of oxygen decreases with increased water temperature, meaning that there would be greater dissolved oxygen available for the rainbow trout than there would be for the zebrafish. Temperature can also affect overall metabolic rate in ectotherms and in particular, teleost fish (Johnston and Dunn, 1987). As body temperature decreases, changes in the enzymatic activity of the cell will produce a reduction in metabolic activity. This reduction in metabolic activity would not only mean a reduction in the detoxification processes of the rainbow trout, but could also reduce the actual uptake and metabolic effects of Se. The high rate of metabolism in the warm-water zebrafish would mean greater metabolic activity and utilization of these seleno-amino acids, such as Se-Met. Future work could consider raising multiple replicates of fish at different temperatures and see how temperature alone can affect Se toxicity.

4.2.3 Bioenergetics

In addition to the cardiovascular effects observed, elevated levels of stored energy (triglycerides and glycogen) were detected in exposed fish. It has been well documented

in lab and field experiments that Se can affect fish energy storage capacity, and my two experiments found similar results. Rainbow trout had significantly greater liver triglyceride and glycogen concentrations in the 15.8 and 47.8 µg Se/g d.m. treatment groups when compared to the controls. While size was limiting the ability to measure liver energy levels in zebrafish, significantly greater whole body glycogen stores were observed in the high exposure group. These elevated energy stores followed trends seen in similar studies (Thomas and Janz, 2011; Thomas et al., 2013; Pettem et al., 2017). These changes could be due to impaired energy homeostasis, caused by modifications of aerobic and metabolic enzyme activities (McGeer et al., 2000; Scott et al., 2002). In order to investigate potential mechanisms of metabolic dysfunction, mRNA transcript abundance of key aerobic energy metabolizing enzymes, citrate synthase (CS) and β-hydroxyacyl coenzyme A dehydrogenase (HOAD) were determined. Citrate synthase is the rate-limiting enzyme in the citric acid cycle and is most often used as an indicator of tissue aerobic scope. In the zebrafish study, elevated dietary Se-Met exposure resulted in significant up-regulation of liver CS mRNA transcript abundance in the 28.8 µg Se/g d.m. treatment group. This was contrasted by the results seen in the rainbow trout study, where a significant down-regulation of liver CS mRNA transcript abundance was seen in the 15.8 and 47.8 µg Se/g d.m. treatment group. HOAD is a key enzyme involved in triglyceride catabolism and a significant down-regulation of both liver and muscle HOAD mRNA transcript abundance was observed in both elevated dietary Se-Met treatments in zebrafish. This was contrasted once again by a significant up-regulation of liver HOAD mRNA transcript abundance in the 47.8 µg Se/g d.m. treatment group in the rainbow trout exposure. While the overall trend of increased energy stores did not change

between species, it is interesting to note these changes seen at the cellular level. This shift at the cellular level could once again bring up the debate between species sensitivity differences vs the effects of exposure temperatures. Temperature would play a pivotal role in overall aerobic and metabolic demand, however no significant differences were seen between species at the overall metabolic level. Further studies should examine expression or activity of intermediary metabolism enzymes such as glycogen phosphorylase and glycogen synthase in Se-Met treated fish to address this question.

4.2.4 Molecular and cellular responses

The goal as toxicologists is to evaluate the overall effects of toxicants and how they may relate to real world applications. While mortalities are sought after data for regulators and risk assessors, this endpoint is too far down the scale of effects. At this point, it is already too late. We should try to avoid relying on pure mortality/survivability results, and instead identify sub-lethal toxicity endpoints that would otherwise go unnoticed until they inevitably stack up and cause lethality. These sub-lethal endpoints can range from morphometric data (length, weights etc.) to behavioural studies, but one of the more up and coming sub-lethal endpoint is looking at gene expression at the cellular level. The cost of gene expression work has drastically dropped and has become readily available to most researchers. By looking at the mRNA abundance in tissues we can see at the cellular level what the organism is mounting for a defense against a potential toxicant. This can be great, but must be taken with a grain of salt, as mRNA transcript abundance is just a “snapshot” of what is happening at the cellular level. Just because an organism has up/down-regulated the abundance of mRNA, doesn’t mean that

it will lead to a 1:1 protein formation. For this reason, mRNA data can be considered insightful, but shouldn't be treated as absolute.

In my thesis, mRNA abundance was used to supplement the metabolic and cardiovascular results seen. It was able to provide insight into what was happening at the cellular level to explain what was observed at the physiological level. As previously mentioned, the leading hypothesis surrounding Se's ability to exert its toxic effects results from Se-induced oxidative stress. Se metabolism leads to the formation of redox reactive intermediates, which react with glutathione and generate toxic hydrogen peroxide, superoxide anions and hydroxyl radicals (Spallholz et al., 1994; Spallholz et al., 2001). When the production of these toxic reactive oxygen species (ROS) overwhelms the endogenous cellular defense mechanisms, oxidative stress will occur. The damage caused by ROS can include lipid peroxidation, DNA oxidation, proteolysis and cellular apoptosis. In order to protect cellular macromolecules from ROS damage, organisms have developed numerous antioxidant defense mechanisms, however exposure to toxic contaminants can overwhelm their abilities leading to an oxidized environment, rather than the desired reduced state. As the cardiovascular system is known to be particularly susceptible to oxidative stress, investigating antioxidant mRNA abundance can be a useful biomarker of oxidative damage caused by contaminant exposure.

In order to investigate this underlying oxidative stress hypothesis, specific mRNA markers of interest were investigated in both adult zebrafish and rainbow trout. mRNA transcript abundance of heart GST-pi class was significantly down-regulated, without any change seen in GPX1A expression in zebrafish exposed to 10.3 µg Se/g d.m. Since catabolism of Se-Met has been reported to cause oxidative stress in fish (Palace et al.,

2004), we would predict an upregulation of GST-pi if the zebrafish heart was able to compensate for oxidative stress. Since GST-pi instead decreased in cardiac tissue, but not liver, this suggests the heart may have been particularly vulnerable to increased damage from reactive oxygen species. Looking at the rainbow trout results reinforces this statement. mRNA transcript abundance of GST, GPX1A and CAT were down-regulated in heart tissues, but upregulated in liver tissues, indicating that the liver may be able to mount protection, while the heart remains susceptible to oxidative stress. Previous studies have shown that oxidative stress has the potential to modify muscle structure and the release of calcium from the sarcoplasmic reticulum (Xia et al., 2004; Musaro et al., 2010). These taken together can impair overall muscle function, including the potential to impair cardiac myofibrils that will lead to altered cardiovascular function. Interestingly, previous studies have shown that oxidative stress can down-regulate the mRNA abundance of protein tyrosine phosphatase 1B (PTB 1B) (Chiarugi, 2005). This reduction was also seen in a zebrafish study using similar Se-Met concentrations, reinforcing the potential for Se to cause oxidative stress (Thomas et al., 2013). This enzyme plays a role in cell migration and angiogenesis (Sallee et al., 2006), but more recently a study has shown that PTB 1B inhibition may also reduce cardiac dysfunction (Thiebaut et al., 2016). If this enzyme inhibition was present in the rainbow trout study, and not in the zebrafish, it could provide insight into what was occurring at the cellular level. Both studies seem to indicate that oxidative stress plays a role in Se toxicity, and while the liver is able to upregulate its protection, the heart remains susceptible. As previously mentioned, mRNA abundance is able to provide some insight but future studies should

investigate the changes at the enzyme activity level following chronic exposure to dietary Se.

4.2.5 Diabetic complications

Although not initially part of my proposal, the link between Se supplementation and the incidence of type 2 diabetes (T2D) captivated my interest. Previous studies from our lab consistently reported elevated energy stores following Se-Met exposure, however proved unable to utilize these stores as shown by a reduced swimming performance (Thomas and Janz, 2011; McPhee and Janz, 2014). In addition, these fish had increases in oxygen consumption rates (MO_2). By evaluating the cardiovascular effects of chronic Se-Met exposure, I was hoping to further shed the light on a sub-lethal Se-induced endpoint. One would expect that the cardiovascular system would respond to an increase in oxygen consumption, by increasing its overall cardiac output. This is normally a linear relationship. However, in Chapter 2, we observed a significant decrease in cardiac output, but consistent with previous studies, an increase in energy stores. Moving to a bigger fish in Chapter 3 allowed me to take non-terminal blood extractions. This opened up a whole new area to investigate. The link between Se supplementation and the incidence of type 2 diabetes (T2D) has been the subject of debate for many years. While some studies indicate Se supplementation diminishes the risk of T2D (Park et al., 2014) and Se-deficiency is associated with insulin resistance (Kljai and Runje, 2001), many other studies are reporting the opposite. We are increasingly seeing in both rodent and human studies, that an excess of Se may impair insulin sensitivity to produce a T2D-like state (Rasekh et al., 1991; McClung et al., 2004; Stranges et al., 2007; Bleys et al., 2007; Laclaustra et al., 2010; Wang et al., 2008; Mueller et al., 2008; Mueller et al., 2009;

Stranges et al., 2010, 2011; Labunsky et al., 2011; Zeng et al., 2012; Liu et al., 2012; Wang et al., 2014). In addition, hyperglycemia is known in mammals to cause excess production of the toxic glucose metabolite, methylglyoxal (Desai et al., 2010; Adolphe et al., 2012; Kalapos, 2013). To our knowledge, this is the first report that methylglyoxal is not only measurable in any fish species, but also that increased levels are associated with hyperglycemia and glucose intolerance, similar to that reported in human diabetics (Desai et al., 2010; Kalapos, 2013). Methylglyoxal, being a reactive carbonyl, also increases oxidative stress as well as covalently modifying macromolecules to cause dysfunction and acting as a precursor for advanced glycation end products that cause cataracts in humans (Desai et al., 2010; Kalapos, 2013). The observation that trout in the highest Se-Met exposure group developed cataracts agrees well with previous studies reporting cataracts in rats exposed to selenite (Shearer et al., 1983) or fish from a high Se environment at Belews Lake, North Carolina (Lemly, 2002).

While diabetes is a human disease term, it is safe to say that Se was able to cause insulin insensitivity or glucose intolerance in these rainbow trout exposed to high doses of Se-Met. Further studies are required.

4.3 Conclusions

In closing, my thesis was able to investigate the sub-lethal effects of chronic dietary Se exposure in two species of fish. The use of two species emphasized the need to characterize selenium's toxicity in a wide range of organisms, as there are substantial differences in species sensitivity to Se. Cardiovascular function and energy homeostasis were investigated and proved to be effective sub-lethal endpoints.

Cardiovascular function was negatively impacted by chronic dietary Se-Met exposure in adult zebrafish, but had opposite results in juvenile rainbow trout. While ventricular fibrosis may be the underlying cause behind the cardiovascular dysfunction seen in zebrafish, an anti-fibrotic protective response was seen in trout. The differences seen here can also open up the discussion regarding warm vs cold water species sensitivity to Se, where future studies should investigate the role of exposure temperature on Se toxicity. Altered energy metabolism was seen in both species with increased stores of glycogen and triglycerides. My thesis was the first to investigate the cardiovascular effects of Se exposure in fish, and proved to be a non-lethal effective sub-lethal endpoint to investigate. Selenium altered cardiovascular function, impaired energy homeostasis, and caused visual and behavioural changes in both fish species. Overall, these effects would alter the aerobic capacity of fish and have potential ecological consequences on overall fitness. Additional research is clearly required to fully understand the biochemical, physiological and cellular mechanisms behind Se toxicity between fish species.

The need to set environmental thresholds and benchmarks is clearly needed for regulators and government agencies. These “tipping points” represent the quantitative critical value, which if crossed, can have serious environmental impacts. The 2016 USEPA whole body tissue guidelines for the protection of aquatic life states a maximum whole body value of 8.5 µg/g d.m. Looking at the results from my thesis, we can see minor effects in adult zebrafish at dietary levels as low as 10.3 µg/g d.m., and yearling rainbow trout at dietary levels as low as 15.8 µg/g d.m. When considering the potential environmental impact, it is always beneficial to set conservative thresholds, however these benchmarks may not represent the true value of risk. Regulators and industries must

adhere to these strict guidelines, regardless if they are realistic. Following the sublethal toxicities seen in my thesis, I believe the 2016 USEPA selenium guidelines represent a safe, realistic criterion for the protection of aquatic life.

The work in my thesis has proven to be very important because sensitivity differences between fish species can lead to an inappropriate Se thresholds. More fish species are clearly needed to be tested, allowing for a wider scope of Se-induced toxicities.

4.4 Future Considerations

My thesis was successful in identifying a novel, sublethal endpoint involved in selenium-induced toxicities. High frequency cardiac biomicroscopy has proven to be an accurate non-invasive way to study fish health *in vivo* without having to sacrifice the test subject. Although the research involved in this thesis was considered as a success, there are areas that could be further investigated.

In Chapter 2, I observed significant cardiotoxicity that was consistent with ventricular fibrosis and reduced cardiovascular performance. Previous work from our lab demonstrated that at similar doses, zebrafish accumulated similar metabolic energy stores. These fish however proved unable to utilize these resources as dictated by a reduced swimming performance and an increase in oxygen consumption following swim tunnel respirometry. While one would assume the fish would respond to an increase in oxygen consumption by increasing the overall cardiac output, this was not seen in our study. This comparison however was done between studies, and future studies should try to do cardiac ultrasonography and swim tunnel respirometry concurrently to have an accurate representation of the sublethal toxicities.

In Chapter 3, the rainbow trout tested at similar Se-Met diets demonstrated opposite cardiovascular results to that observed in the zebrafish. Comparing these two fish species demonstrated the vast species sensitivity differences Se can have. While the zebrafish is a warm water fish, tested at 28 °C, the rainbow trout is a cold-water fish, tested at 12 °C. While this significant 16 °C change in testing temperature could the sole reason for the significant changes in cardiovascular results, overall body size could have also played a part. While zebrafish are one of the most sensitive fish species, they are also one of the smallest in size. Future research should consider a range in temperature testing conditions to determine if temperature may play a factor in overall Se toxicity. As rainbow trout are known to be tolerant to temperatures as low as 9 °C and as high as 22 °C (Raleigh et al. 1984), one could potentially test this species at incremental temperatures and measure the uptake and of Se.

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